Structure-Function Analysis of the Conserved Histone Chaperone Spt6

Structure-Function Analysis of the Conserved Histone Chaperone Spt6

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Abstract

Chromatin structure is crucial to regulate access to the genome for processes such as transcription, recombination, DNA repair, and DNA replication. Spt6, a key factor involved in regulating chromatin structure, is conserved throughout eukaryotes. Spt6 has been shown to function in many aspects of gene expression, including nucleosome assembly, transcription initiation and elongation, and mRNA processing and export. In addition, Spt6 has several conserved domains; however, little is known about their functions. I have performed a structure-function analysis of Spt6 using three separate approaches. First, I employed a random insertion mutagenesis that has identified sixty-seven mutants. While these mutants did not provide information regarding known domains, some have phenotypes that may prove useful for future study. Second, in a collaborative project with the Romier lab, I studied the functional roles of the Spt6 SH2 domains. I have shown that deletion of the region of *SPT6* encoding the SH2 domains causes severe mutant phenotypes without affecting Spt6 protein levels, demonstrating the importance of the SH2 domains of Spt6. Third, in an additional collaboration with the Romier lab, I showed that mutations that alter the region of Spt6 that interacts with the conserved transcription factor Spn1 impair Spt6 functions *in vivo*. Overall, this multi-pronged structure-function analysis of Spt6 has provided new insights into the tandem SH2

domains of Spt6, the Spt6-Spn1 interaction, and the uses and limitations of insertion mutagenesis.

In addition, I have attempted to explore a possible role for Spt6 in transcription-associated mutagenesis. After employing several types of *in vivo* assays, I conclude that a possible role for Spt6 in transcription-associated mutagenesis is uncertain, as the results (with respect to a role for Spt6) reproducibly vary depending on the assay used. Thus, understanding this aspect of Spt6 biology awaits better assays and understanding of transcription-associated mutagenesis. Overall, the work in this dissertation will serve to further elucidate the mechanisms of Spt6 in chromatin regulation, transcription, and DNA damage repair.

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Dedication

"There are only two ways to live your life. One is as though nothing is a miracle. The other is as though everything is a miracle."

~ *Albert Einstein*

This dissertation is dedicated to everyone who has ever looked in wonder at the biology of the natural world and been amazed by its intricacy and miraculous beauty and wanted to learn more about it.

Acknowledgments

"I can no other answer make but thanks,

And thanks, and ever thanks..."

~ *Sebastian, Twelfth Night, Act 3, scene 3*

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Most importantly, I would like to thank my family who has always been there for me no matter the time of day or the distance between us.

I love you more than words can say.

Chapter 1

Introduction

I. Chromatin and Transcription: A Brief Summary

All eukaryotic organisms face the challenge of compacting their lengthy genomes within a microscopic nucleus. For example, the human genome is approximately two meters in length when stretched out end-to-end; yet this must fit into a human nucleus 6 μm in diameter (Campbell et al., 1997). This is geometrically equivalent to packing 24 miles of extremely fine thread into a tennis ball (M. Murawska, unpublished calculation). Furthermore, this compaction must be accomplished in an organized fashion to allow normal chromosomal processes, such as replication, transcription, and segregation to occur. To help to accomplish this feat, eukaryotic organisms employ nucleosomes, each composed of 147 base pairs (bp) of DNA wrapped around a histone protein octamer containing two copies each of histones H2A, H2B, H3, and H4 (Lee and Workman, 2007; Reinberg and Sims, 2006; Svejstrup, 2003). In the context of transcription, however, a dilemma arises: how can RNA polymerase II (RNAPII) traverse the obstacle course of nucleosomes? Here I provide an overview of transcription and chromatin as a backdrop to understand Spt6, the histone chaperone that is the focus of this dissertation.

Many key advances in understanding transcription and chromatin have been made in the budding yeast *Saccharomyces cerevisiae* so this section deals primarily with yeast gene expression (Hahn and Young, 2011). However, these processes are highly conserved throughout eukaryotes so the transcription and chromatin processes described in this section are typical for what is found in most eukaryotes (Botstein and Fink, 2011; Rando and Winston, 2012).

Transcription Overview

In budding yeast, the transcription initiation process begins at the promoter sequence, which is generally located in a nucleosome-free region (Albert et al., 2007; Hahn and Young, 2011; Kaplan et al., 2009; Kent et al., 2011; Lee et al., 2007; Mavrich et al., 2008; Yuan et al., 2005). Nucleosome-free regions are hypothesized to allow unimpeded transcription factor access and are flanked downstream by a strongly positioned +1 nucleosome (Albert et al., 2007; Hahn and Young, 2011; Kaplan et al., 2009; Kent et al., 2011; Lee et al., 2007; Mavrich et al., 2008; Yuan et al., 2005).

Subsequently, activators bind and facilitate binding of coactivators such as SAGA and Swi/Snf (Hassan et al., 2001; Hassan et al., 2002; Rando and Winston, 2012; Workman, 2006; Yudkovsky et al., 1999). Nearby histones are acetylated, increasing nucleosome mobility and facilitating binding with bromodomain-containing proteins such as those within SAGA and Swi/Snf (Dhalluin et al., 1999; Hassan et al., 2001; Hassan et al., 2002; Rando and Winston, 2012; Workman, 2006). A preinitiation complex (PIC) containing TATA-binding protein (TBP) and a set of general transcription factors including TFIIA, TFIIB, and TFIIF is formed at the TATA box (Hahn and Young, 2011; Thomas and Chiang, 2006). TFIIB and TFIIF assist in recruiting RNAPII to promoter sequences, as well as facilitating initiation activity and start site recognition (Hahn and Young, 2011). Additional factors TFIIE and TFIIH assist with promoter DNA strand separation and facilitate the shift to active transcription (Hahn and Young, 2011). Subsequently, RNAPII is phosphorylated on its C-terminal domain (CTD) at serine-5 (S5p) and -7 (S7p) as transcription initiates (Hahn and Young, 2011; Workman, 2006).

Next, RNAPII begins to elongate across the open reading frame (ORF) of the gene with the assistance of many elongation factors including Swi/Snf, RSC, SAGA, and NuA4 (Carey et al., 2006; Ginsburg et al., 2009; Hahn and Young, 2011; Mas et al., 2009; Rando and Winston, 2012; Schwabish and Struhl, 2007; Wyce et al., 2007). During elongation, the RNAPII CTD is phosphorylated at serine-2 (S2p) and tyrosine-1 (T1p) (Hahn and Young, 2011; Mayer et al., 2012; Workman, 2006). RNAPII CTD T1p helps prevent binding of termination factors such as Nrd1, Pcf11, and Rtt103 during transcription elongation (Mayer et al., 2012). At the polyadenylation site at the end of the ORF, RNAPII CTD T1p levels drop, while CTD S2p levels remain high and help recruit factors involved in mRNA 3'-end processing and termination (Mayer et al., 2012). After successful mRNA synthesis, transcription concludes with termination and disassociation of RNAPII from the DNA template (Shandilya and Roberts, 2012).

Summary of Chromatin Alterations during Transcription

As one might imagine, chromatin is altered significantly during the process of transcription. In general, the level of transcription correlates with the level of nucleosome loss over promoters and coding regions (Field et al., 2008; Jiang and Pugh, 2009; Radman-Livaja and Rando, 2010; Schwabish and Struhl, 2007; Shivaswamy et al., 2008; Weiner et al., 2010; Zawadzki et al., 2009). Nucleosomes are evicted from promoters by both transcription factors and ATP-dependent remodelers such as Swi/Snf and RSC (Rando and Winston, 2012). In addition, RNAPII passage itself can cause changes in nucleosome occupancy and position (Weiner et al., 2010). Interestingly, RNAPII is capable of passing through chromatin *in vitro* without disassociating nucleosomes from the DNA (Kulaeva et

al., 2009; Kulaeva et al., 2010). Further *in vitro* studies have suggested that the first round of transcription removes one H2A/H2B dimer while subsequent rounds of transcription remove the remaining histone hexamer (Chang et al., 2014; Jin et al., 2010; Kulaeva et al., 2009; Kulaeva et al., 2007; Kulaeva et al., 2010). Overall, a high level of RNAPII transcription leaves altered chromatin and evicted nucleosomes in its wake that must be reassembled.

Nucleosome reassembly following transcription is crucial for proper gene regulation, cell survival, and normal aging (Chang et al., 2014; Cheung et al., 2008; Feser et al., 2010; Martens et al., 2005). Nucleosome reassembly is accomplished by the action of histone chaperones, generally defined as factors that interact with chromatin *in vivo*, bind nucleosomes *in vitro*, and assist in histone eviction, exchange, or deposition (Rando and Winston, 2012).

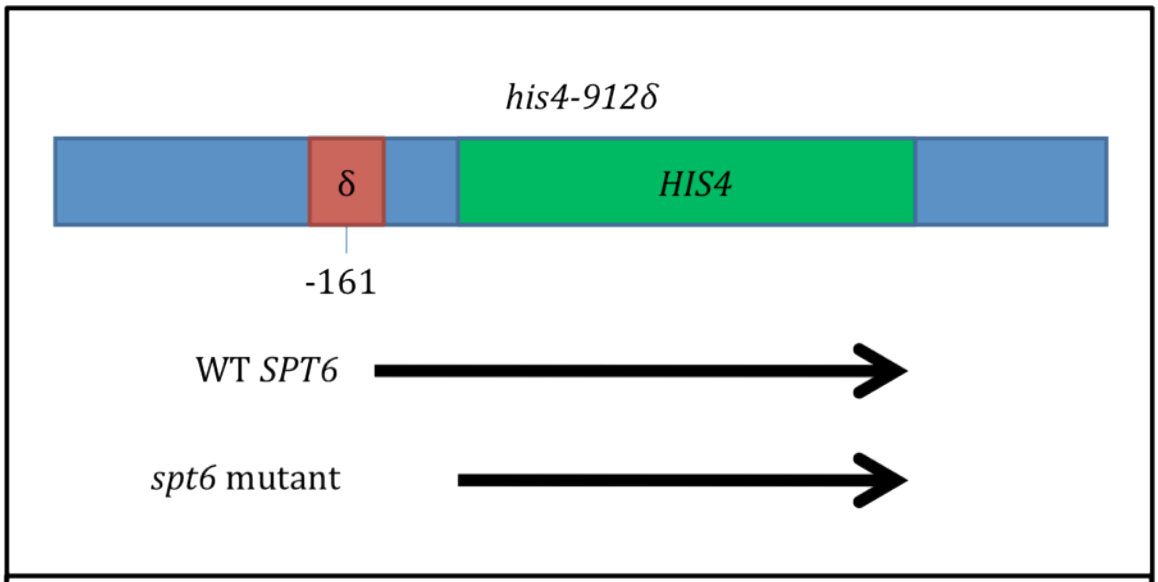
One of the proteins believed to be crucial for nucleosome reassembly is Spt6, which colocalizes with elongating RNAPII (Adkins and Tyler, 2006; Andrulis et al., 2000; Bortvin and Winston, 1996; Endoh et al., 2004; Kaplan et al., 2000; Kim et al., 2004; Mayer et al., 2012; Ni et al., 2008; Saunders et al., 2003; Zobeck et al., 2010). Spt6 has diverse roles in transcription and chromatin regulation including acting as a histone chaperone (discussed below). Spt6 shares many mutant phenotypes with the FACT complex, another histone chaperone, which is composed of Spt16 and Pob3 (Brewster et al., 2001; Cheung et al., 2008; Formosa et al., 2002; Kaplan et al., 2003; Malone et al., 1991; Mason and Struhl, 2003). Although the mechanism of FACT has been extensively characterized (Rando and Winston, 2012), the precise role of Spt6 in the intertwined processes of transcription and chromatin structure is still unclear. Therefore, Spt6 was the focus of my dissertation.

II. Spt6

Discovery of Spt6

Mutations in *SPT6* were originally identified as suppressors of a δ (the long terminal repeat of a Ty transposable element) insertion mutation in the promoter of the *S. cerevisiae HIS4* gene (Figure 1-1) (Winston et al., 1984). The *spt6-140* mutant allele (Table 1-1) allowed for normal transcription of the *HIS4* gene despite the presence of the 330 bp δ insertion within the *HIS4* promoter (Clark-Adams and Winston, 1987). Restoration of functional *HIS4* transcription despite the continued presence of a δ insertion is referred to as an Spt$^-$ phenotype. The *spt6* mutants were also temperature-sensitive for growth (Ts$^-$) at 37°C, suggesting that *SPT6* is essential for viability (Winston et al., 1984).

Mutations in *SPT6* were subsequently found in a number of other yeast mutant hunts for suppressors of transcriptional defects. In one such mutant hunt, work by the Carlson lab isolated several suppressors of a *snf2* mutation (Neigeborn et al., 1986). *SNF2* is required for normal derepression of *SUC2* (which encodes invertase) under glucose deprivation conditions (Neigeborn and Carlson, 1984). The *snf2* suppressors, located within a gene called *SSN20* (**S**uppressor of ***sn****f2*), partially restore *SUC2* expression in a *snf2* background (Neigeborn et al., 1986). Further characterization revealed that *SSN20* is the same gene as *SPT6* (Neigeborn et al., 1987). Similarly, the *CRE2* gene (**C**atabolite **R**epressor **E**lement), later shown to be allelic with *SPT6*, was identified in a hunt for mutants that allowed *ADH2* (encodes for glucose-repressible alcohol dehydrogenase) expression under glucose-repressed conditions (Denis, 1984; Denis and Malvar, 1990). Finally, the *SPT6* gene was also identified by *bur* mutations (**B**ypass **U**pstream activating sequence (UAS)



Strain	*HIS4* transcript	Growth on SC-His	Phenotype
WT *SPT6*	Nonfunctional	-	Spt^+
spt6 mutant	Functional	+	Spt^-

Figure 1-1: Discovery of *SPT6* through selection for Ty δ insertion suppressors.

Ty912 is a yeast transposable element with an internal region flanked by two long terminal repeats (δ sequences). Insertion of a δ (red) into the *HIS4* promoter leads to a nonfunctional transcript and inability to grow on medium lacking histidine (SC-His). Mutants of *SPT6* suppress δ insertions and restore a functional *HIS4* transcript. The mRNA transcripts produced in wild-type and *spt6* mutant yeast are shown by black arrows. Mutations in *spt6* appear to affect the balance between the production of these two transcripts, favoring the shorter functional *HIS4* transcript. (Swanson et al., 1991; Winston et al., 1984; Yamaguchi et al., 2001)

Table 1-1: List of well-studied *spt6* mutant alleles mentioned in this dissertation. For more information regarding Spt6 domains, see Figure 1-2 and Table 1-2. (Abbreviations: Ts$^-$, temperature sensitive at 37°C; Cs$^-$, cold sensitive at 16°C; HU^S, Hydroxyurea sensitive; 6-AU^S, 6-Azauracil sensitive; Phleo^S, Phleomycin sensitive; aa, amino acids) (Data from Kaplan, 2002; Kaplan et al., 2005; Chu et al., 2006; E. Loeliger, unpublished data)

***spt6* allele**	**Change in Spt6 protein caused by mutation**	**Phenotypes**
spt6-140	L653P	Ts$^-$, Spt$^-$, cryptic initiation
spt6-50	K1274Stop, last 177 aa removed	Ts$^-$, Cs$^-$, Spt$^-$, cryptic initiation, slow growth, HU^S, 6-AU^S
spt6-1004	aa 931-994 deleted (HhH domain deleted)	Ts$^-$, Spt$^-$, cryptic initiation, Phleo^S, histone modification defects, synthetically lethal when combined with deletions of genes encoding Paf1 complex components
spt6-1002	aa 2-122 deleted	Ts$^-$, Spt$^-$, cryptic initiation, slow growth, synthetically lethal when combined with deletions of genes encoding Paf1 complex components

Requirement) that allow increased transcription of *SUC2*, despite the deletion of the *SUC2* UAS normally required for *SUC2* expression (Prelich and Winston, 1993).

Overall, the multiple modes of discovery of Spt6 all pointed to the importance of Spt6 in transcription regulation. Spt6 was later found to be an essential nuclear protein conserved throughout eukaryotes (Chiang et al., 1996; Clark-Adams and Winston, 1987; Kaplan et al., 2000; Keegan et al., 2002; Neigeborn et al., 1987; Swanson et al., 1990).

Biological Roles of Spt6

The Roles of Spt6 in Transcription Regulation

Given that mutations in *SPT6* were found to be suppressors of transcriptional defects, Spt6 was postulated to have a role in transcription regulation (Denis, 1984; Denis and Malvar, 1990; Neigeborn et al., 1987; Neigeborn et al., 1986; Prelich and Winston, 1993; Yamaguchi et al., 2001). Work in *S. cerevisiae* showed that the *spt6-50* mutant allele (Table 1-1) was sensitive to 6-azauracil, also suggesting a role in transcription elongation (Hartzog et al., 1998). Consistent with this, Spt6 interacts genetically with the transcription elongation factor TFIIS (for additional information, see Appendix 2) and interacts physically with the transcription elongation complex Spt4/Spt5 (Hartzog et al., 1998; Lindstrom et al., 2003; Swanson and Winston, 1992). Importantly, Spt6 binds the phosphorylated CTD of RNAPII and co-localizes with RNAPII along transcriptionally-active genes in a number of model organisms, providing further evidence that Spt6 functions in transcription elongation (Andrulis et al., 2000; Diebold et al., 2010b; Endoh et al., 2004; Ivanovska et al., 2011; Kaplan et al., 2005; Kaplan et al., 2000; Kim et al., 2004; Liu et al., 2011; Ni et al., 2008; Saunders et al., 2003; Sun et al., 2010; Yoh et al., 2007; Zobeck et al.,

2010). In fact, biochemical work indicates that human Spt6 enhances the elongation rate of RNAPII *in vitro* and *in vivo* (Ardehali et al., 2009; Endoh et al., 2004). Taken together, these results provide strong evidence for a role of Spt6 in transcription elongation.

More recently, RNA-seq analysis in the fission yeast *Schizosaccharomyces pombe* has demonstrated that *spt6* mutants cause drastic changes in transcription genome-wide (Degennaro et al., 2013). This includes elevated antisense transcription in >70% of all genes, changes in levels and start sites of mRNA, and increased intragenic transcription (described more below) (Degennaro et al., 2013). Similar elevated antisense transcription was seen in *S. cerevisiae spt6* mutants, equivalent to the transcription alterations seen upon histone protein H4 depletion (van Bakel et al., 2013). The H3K36 methyltransferase, Set2, as well as chromatin remodelers, Isw1 and Chd1, are required for repressing this antisense transcription (Degennaro et al., 2013; van Bakel et al., 2013). Also, in addition to binding S2p phosphorylated RNAPII CTD, Spt6 appears to be required for S2p itself through associating with and stabilizing Ctk1 (the major serine-2 CTD kinase) (Dronamraju and Strahl, 2014). Spt6 may also be important for RNAPI transcription since there is physical and genetic evidence for an interaction between Spt6 and Rpa43, a subunit of RNAPI (Beckouet et al., 2011). Altogether, this work shows that Spt6 may have more far-reaching effects on transcription than was previously thought.

Interestingly, *spt6* mutants cause a transcriptional defect known as cryptic intragenic initiation, where transcription initiates from internal promoters within ORFs, leading to short transcripts (Cheung et al., 2008; Kaplan et al., 2003). Consistent with this, mutations in *SPT6* are known to impair histone H3K36 methylation and recruitment of the transcription factor Spt2; both transcriptional features that contribute to repression of

cryptic initiation (Carrozza et al., 2005; Chu et al., 2006; Nourani et al., 2006; Youdell et al., 2008). Cryptic initiation is observed in a number of other mutants including those with mutations in *SPT2, SPT4, SPT5, SPT16,* and *SET2*, indicating that this phenomenon is not unique to *spt6* mutants (Cheung et al., 2008). In particular, *SET2* and H3K36 trimethylation play a prominent role in repression of cryptic transcription (Carrozza et al., 2005; Cheung et al., 2008; Chu et al., 2006; Degennaro et al., 2013; Li et al., 2007; Yoh et al., 2008; Youdell et al., 2008).

It is now clear that cryptic transcription is a pervasive phenomenon (Cheung et al., 2008). Microarray data has demonstrated that *spt6* mutants cause cryptic transcription in over 1000 yeast genes (approximately 1/6 of the yeast genome) (Cheung et al., 2008). Some of these cryptic transcripts are even produced in wild-type yeast following a nutritional shift (Cheung et al., 2008). Intriguingly, a number of these cryptic transcripts are translated to produce proteins (Cheung et al., 2008). Hypotheses for the function of cryptic transcripts include 1) a mechanism for generating alternative gene products, akin to alternative splicing, 2) a mechanism for facilitating evolutionary genetic changes, 3) regulation of transcription or chromatin structure without producing a functional transcript, and 4) transcriptional "noise" with no apparent biological role (Cheung et al., 2008). Only a handful of examples of functional cryptic transcripts have been described (Bickel and Morris, 2006; Ono et al., 2005). In these cases, the individual cryptic transcripts examined were shown to have roles in termination suppression (Ono et al., 2005) and response to mating pheromone (Bickel and Morris, 2006). However, beyond a few isolated examples, the physiological importance of cryptic transcripts remains elusive.

For the purposes of my thesis work, it should be noted that cryptic initiation can be easily monitored in *S. cerevisiae* using a *FLO8-HIS3*-based system where the *HIS3* ORF is inserted 3' of the *FLO8* cryptic transcript start site (Cheung et al., 2008). In this reporter, the *HIS3* coding sequence is inserted out-of-frame with respect to the full-length *FLO8* coding sequence (Cheung et al., 2008). Therefore, *HIS3* transcript can only be produced by transcription initiation at the cryptic start site and can be detected by yeast growth on medium lacking histidine (SC-His). This reporter has facilitated characterization of cryptic transcription by providing a reliable and convenient technique for cryptic transcription detection in a wide range of mutants.

The Roles of Spt6 in Chromatin Regulation

Spt6 appears to also have a crucial role in regulation of chromatin structure. The demonstration that Swi/Snf controls chromatin structure *in vivo* (Hirschhorn et al., 1992), combined with the suppression of *snf2* mutations by *spt6* mutations (Neigeborn et al., 1986), suggested that Spt6 might also control chromatin structure. Subsequent work confirmed that *spt6* mutations suppress *snf2* mutations at the level of chromatin structure (Bortvin and Winston, 1996). This work also demonstrated that Spt6 binds directly to histones H3 and H4 from yeast and humans, and is capable of assembling histones onto naked DNA *in vitro* (Bortvin and Winston, 1996). *In vivo*, Spt6 is required for histone reassembly onto promoters to confer transcriptional repression (Adkins and Tyler, 2006). Mutations in *SPT6* lead to increased MNase sensitivity over some transcribed regions, presumably from histone loss (Bortvin and Winston, 1996; Ivanovska et al., 2011; Kaplan et al., 2003).

The control of chromatin structure by Spt6 is also apparent from the finding that, in both *S. cerevisiae* and *S. pombe*, an *spt6* mutation causes nucleosome loss over many coding regions, preferentially over highly transcribed genes (Degennaro et al., 2013; Ivanovska et al., 2011; van Bakel et al., 2013). This result is consistent with a model in which nucleosomes are more frequently lost from highly transcribed genes and Spt6 is required for their reassembly. Unexpectedly, in *S. cerevisiae*, the *spt6* effects on nucleosome loss did not correlate with *spt6* effects on mRNA levels and cryptic transcription, suggesting that the effect of Spt6 on chromatin regulation is independent of its effect on transcription (Ivanovska et al., 2011).

Spt6 appears to also play a crucial role in nucleosome phasing and patterning. Spt6 is important for +1 nucleosome positioning at the *CHA1* locus (Ivanovska et al., 2011) and for establishing 5' and 3' nucleosome-depleted regions genome-wide (Perales et al., 2013). In fact, mutations in *SPT6* cause almost complete loss of nucleosome patterning over genes in *S. cerevisiae* and *S. pombe*, particularly in the first 500 bp (Degennaro et al., 2013; Ivanovska et al., 2011; Perales et al., 2013; van Bakel et al., 2013). Interestingly, based on promoter swapping experiments in *S. cerevisiae*, it appears that the effects of an *spt6* mutant on histone loss are determined by the promoter more so than the gene body (Perales et al., 2013). Overall, *spt6* mutants have drastically altered nucleosome positioning with nearly complete loss of nucleosome patterning over promoters and coding regions.

Spt6 also affects histone turnover over heterochromatic loci in *S. pombe*. By preventing transcription-coupled histone turnover, Spt6 maintains post-translational modifications at histone H3 and preserves epigenetic integrity (Kato et al., 2013). A genetic

interaction between Spt6 and Clr3 (a histone deacetylase) is suggested by increased H3K56 acetylation at the pericentromere in the *spt6 clr3* double mutant compared to the *spt6* or *clr3* single mutants, suggesting that these regulators of histone turnover have redundant mechanisms and act in parallel at the pericentromere (Kato et al., 2013). Taken together, these results suggest that Spt6 affects histone turnover in *S. pombe*, acting in parallel with Clr3.

Spt6 appears to also regulate chromatin indirectly through interaction with Spt2 (Bhat et al., 2013). Spt2 helps inhibit spurious transcription in yeast and maintains nucleosome levels in coding regions (Nourani et al., 2006; Smolle and Workman, 2013; Smolle et al., 2013; Thebault et al., 2011). Spt6 physically interacts with Spt2 in a Casein Kinase 2 (CK2)-dependent fashion (Bhat et al., 2013). Spt6 recruits Spt2 to areas of active transcription and it is hypothesized that Spt2 and CK2 may regulate the dynamics of Spt6 association and disassociation from nucleosomes (Bhat et al., 2013; Thebault et al., 2011). Overall, the Spt2-Spt6 interaction may be partially responsible for the effects of Spt6 in regard to preventing cryptic transcription and nucleosome loss over ORFs.

The Roles of Spt6 in Histone Modifications

A core role of Spt6 in chromatin regulation, which likely accounts for some of its effects on cryptic transcription, is its requirement for particular histone modifications. The most well-established of these is the requirement of Spt6 for H3K36 trimethylation, a histone modification important for repressing cryptic initiation and associated with active transcription over 3' regions (Carrozza et al., 2005; Cheung et al., 2008; Chu et al., 2006; Joshi and Struhl, 2005; Youdell et al., 2008). The requirement of Spt6 for H3K36

trimethylation is conserved across *S. pombe*, *S. cerevisiae*, and humans (Carrozza et al., 2005; Chu et al., 2006; Degennaro et al., 2013; Yoh et al., 2008; Youdell et al., 2008). In *S. pombe*, Spt6 is required for maximum recruitment of Set2 (the H3K36 methyltransferase) which partially explains the H3K36 trimethylation defect in *spt6* mutants (Degennaro et al., 2013). On the other hand, in an *S. cerevisiae spt6* mutant, the H3K36 trimethylation defect is partially due to decreased Set2 protein levels (Youdell et al., 2008), whereas there are normal Set2 levels in an *S. pombe spt6* mutant (Degennaro et al., 2013). In humans, the Spt6-mediated H3K36 trimethylation defect is partially responsible for the abnormalities in immunoglobulin locus class switch recombination observed in *spt6* mutants (discussed below) (Begum et al., 2012).

Another important histone modification affected by Spt6 is H3K4 trimethylation, associated with active transcription at the 5' end of transcribed regions (Smolle and Workman, 2013). Interestingly, Spt6 is required for normal levels of H3K4 trimethylation in *S. pombe* (Degennaro et al., 2013; Kato et al., 2013) and humans (Begum et al., 2012), but not in *S. cerevisiae* (M. Murawska, N. Reim, and F. Winston, unpublished data). In *S. pombe*, *spt6* mutations cause a dramatic loss of all detectable H3K4 trimethylation, mostly due to a defect in recruitment of the COMPASS complex, which is required for H3K4 trimethylation (Degennaro et al., 2013). In humans, similar to H3K36 trimethylation, one of the downstream effects of loss of H3K4 trimethylation is abnormal immunoglobulin locus class switch recombination (discussed below) (Begum et al., 2012).

Studies of histone modifications in *S. pombe* heterochromatin have proven to be particularly useful because of the similarities of its heterochromatin to the heterochromatin of larger eukaryotes (Grewal, 2010; Kiely et al., 2011; Moazed et al.,

2006). In this context, mutations in *SPT6* result in a large decrease in H3K9 trimethylation (a histone modification commonly seen in *S. pombe* heterochromatin silencing mutants), possibly through decreased recruitment of the CLRC complex, which is required for H3K9 trimethylation (Kiely et al., 2011). The effects on H3K9 dimethylation differ between studies, with one group showing no requirement of Spt6 for H3K9 dimethylation (Kiely et al., 2011) and another observing H3K9 dimethylation defects in an *spt6* mutant (Kato et al., 2013). This discrepancy is most likely due to the different *spt6* mutant alleles used: the allele used by Kiely et al encoded a mutant protein lacking the Spt6 helix-hairpin-helix (HhH) domain while Kato et al utilized a deletion mutant lacking the YqgF RNase-like domain (Spt6 domains discussed below) (Kato et al., 2013; Kiely et al., 2011).

In addition, H3K14 acetylation was increased four-fold in an *spt6* mutant, which may partially account for the severe heterochromatin silencing defect observed in *S. pombe spt6* mutants (Kiely et al., 2011). In regard to this, an *spt6* mutation disrupts silencing at all heterochromatic loci (the pericentric repeats, silent mating type locus, and subtelomeric regions), in addition to affecting centromere function (Kiely et al., 2011). Overall, mutations in *SPT6* drastically alter the histone modification landscape over heterochromatin, which leads to heterochromatin silencing defects in fission yeast.

In zebrafish and mammals, Spt6 has been found to counteract the level of another histone modification, H3K27 trimethylation. H3K27 trimethylation is a histone mark important for transcription repression and deposited by the Polycomb Repressive Complex 2 (PRC2) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Wang et al., 2013). Spt6-mediated H3K27 demethylation is required for activation of the normal myogenic gene program which includes MyoD and other muscle-specific gene

expression and cell differentiation (Wang et al., 2013). The mechanism for H3K27 demethylation appears to be Spt6-mediated chromatin association of the H3K27 demethylase, KDM6A (UTX) (Wang et al., 2013). Spt6 also physically interacts with JMJD3, an H3K27 demethylase, which helps recruit Spt6 to JMJD3-targeted regions of the genome (Chen et al., 2012). In conclusion, Spt6-mediated recruitment of H3K27 demethylases is crucial for the H3K27 demethylation required for normal myogenesis.

Spt6 Localization Pattern on Chromatin

Given the crucial role of Spt6 in chromatin and transcription regulation, determining its localization pattern on chromatin is an important starting point for understanding Spt6 function. Work from our lab and others, using *Drosophila melanogaster* polytene chromsome staining (Andrulis et al., 2000; Kaplan et al., 2000) and *S. cerevisiae* ChIP-chip (Ivanovska et al., 2011), showed that Spt6 recruitment correlates with transcription levels and with levels of phosphorylated, actively transcribing RNAPII. Spt6, mirroring the recruitment pattern of fellow transcription factors Spt4 and Spt5, is not present at promoters but Spt6 occupancy increases sharply downstream of the 5' transcription start site and is maintained throughout the transcribed region (Mayer et al., 2010). This is in contrast to Spn1/Iws1, the conserved binding partner of Spt6 (described in a later section), which is mostly found in high levels at the 3' end of genes (Mayer et al., 2010). One key unanswered question is why the binding partners, Spt6 and Spn1, have different genomic localization patterns. The chromatin recruitment profile of elongation factors, such as Spt6 and Spn1, is distinct from those of initiation or termination factors (Mayer et al., 2010).

Overall, these findings demonstrate that Spt6 binds throughout chromatin and that its recruitment correlates with transcription and RNAPII levels.

Spt6 has Roles in mRNA Export and Processing

Spt6 has also been shown to be important for other processes associated with transcription. Microarray data from *spt6* mutants show a profile of transcription which clusters with that of mutants involved in mRNA export and processing (Burckin et al., 2005). Consistent with this result, experiments in *D. melanogaster* suggest a physical interaction between Spt6 and members of the exosome complex, a 3'-5' exoribonuclease involved in processing of structural RNA and in degradation of improperly processed, spliced or adenylated pre-mRNA (Andrulis et al., 2002; Bousquet-Antonelli et al., 2000; Butler, 2002; Hilleren et al., 2001; Torchet et al., 2002; van Hoof et al., 2002). Spt6 also co-purifies with the mRNA capping enzyme and cap methyltransferase in budding yeast (Lindstrom et al., 2003). Mutations in *SPT6* affect mRNA 3'-end formation and cause read-through of the polyA tail (Kaplan et al., 2005). In humans, *spt6* mutants lead to splicing defects in select mRNAs, while ectopic *SPT6* expression leads to nuclear retention of bulk poly(A)$^+$ RNAs (Yoh et al., 2008). Taken together, these studies suggest that Spt6 may connect transcription elongation to downstream or simultaneous mRNA processing and export steps.

The Role of Spt6 in Metazoan Development and Signal Transduction

Spt6 has been implicated in many areas of biology in diverse organisms from yeast to human. Spt6 is essential for *D. melanogaster* development (Ardehali et al., 2009),

Caenorhabditis elegans gut development (Nishiwaki and Miwa, 1998; Nishiwaki et al., 1993), zebrafish development including somitogenesis and cardiac differentiation (Keegan et al., 2002; Kok et al., 2007; Serluca, 2008; Wang et al., 2013), signal transduction in metazoans (Baniahmad et al., 1995; Shen et al., 2009; Yoh et al., 2007), mammalian and zebrafish muscle development (Wang et al., 2013), and Human Immunodeficiency Virus type 1 (HIV-1) and Human Cytomegalovirus (HCMV) viral genome transcription (Cygnar et al., 2012; Gallastegui et al., 2011; Nakamura et al., 2012; Vanti et al., 2009; Winkler et al., 2000; Yoh et al., 2007). In addition, recent studies suggest that Spt6 is required for germline stem cell maintenance in *D. melanogaster* (Neumuller et al., 2012). These extensive effects on development, signal transduction, and viral transcription illustrate the far-reaching consequences of the role of Spt6 in transcription regulation and chromatin structure.

The Role of Spt6 in HIV-1 and HCMV Viral Transcription

Some studies have suggested important roles for Spt6 in HIV-1 transcription. Spt6 is believed to regulate chromatin over regulatory elements of the latent HIV-1 genome and may contribute to repressing reactivation of latent virus and preventing illness progression (Vanti et al., 2009). Interaction with PAAF1 (a proteasome-associated factor) is required to prevent degradation of Spt6, which otherwise causes aberrant cryptic initiation over the HIV-1 viral genome (Nakamura et al., 2012). In one case, depletion of Spt6 promoted HIV-1 promoter reactivation, concurrently with increased chromatin accessibility to MNase digest over the viral genome and regulatory regions (Gallastegui et al., 2011). Some have

hypothesized that Spt6 may act to maintain HIV-1 proviral latency (Gallastegui et al., 2011; Nakamura et al., 2012; Vanti et al., 2009).

In addition, during HCMV infection, Spt6 is required for efficient viral replication (Cygnar et al., 2012; Winkler et al., 2000). The C-terminal region of human Spt6 binds the HCMV protein pUL69 in a region conserved among herpesviruses, and this interaction appears to be required for efficient viral gene expression (Winkler et al., 2000). Interestingly, this Spt6 C-terminal region also binds histone H3 in humans, and pUL69 can inhibit this Spt6-H3 interaction (Winkler et al., 2000). Based on this, HCMV pUL69 is believed to prevent Spt6-H3 binding, resulting in loss of human Spt6 chromatin remodeling functions and allowing efficient HCMV viral replication (Winkler et al., 2000). Overall, this work suggests Spt6 has an important role in HIV-1 and HCMV viral infection in humans.

A Role for Spt6 in Antibody Diversification in Mammals

Consistent with its central role in chromatin regulation, Spt6-dependent histone modifications are important for the normal function of the mammalian immune system. Immunoglobulin genes in antigen-stimulated B-lymphocytes are diversified to detect a wide variety of targets and pathogens by two different genetic alteration mechanisms: class-switch recombination (CSR) and somatic hypermutation (SHM) (Honjo et al., 2002; Kinoshita and Honjo, 2001). CSR results in switching between different immunoglobulin heavy chain constant region genes while SHM introduces random point mutations into the immunoglobulin gene variable region (Honjo et al., 2002; Kinoshita and Honjo, 2001). CSR and SHM both require activation-induced cytidine deaminase (AID) which is specifically expressed in B-lymphocytes and initiates single-stranded DNA breaks crucial for CSR and

SHM (Muramatsu et al., 2007).

Recent studies have shown that Spt6 is required both for normal CSR and SHM, and Spt6 regulates H3K4 trimethylation marks across CSR and SHM target loci (Begum et al., 2012; Okazaki et al., 2011). In addition, depletion of Spt6 decreases H3K4 and H3K36 trimethylation (but not H3 levels) across the gene encoding AID and subsequently blocks AID-induced DNA cleavage required for CSR and SHM (Begum et al., 2012; Okazaki et al., 2011). Interestingly, the C-terminal tandem SH2 domains of Spt6 (discussed below) play a role in normal CSR, as Spt6 truncations missing this region are unable to restore normal CSR and H3K4 trimethylation levels, and also result in a defect in recruitment of the histone methyltransferase Set1A over AID-targeted loci (Begum et al., 2012). Overall, Spt6 clearly plays a crucial role in regulating diversification of antibodies in mammalian B-lymphocytes, partially through regulation of H3K4 and H3K36 trimethylation and Set1A recruitment.

Domain Structure of Spt6

Although several functions of Spt6 have been described, little is known about whether these functions are associated with a particular domain of the Spt6 protein. This is an important issue investigated in Chapter 2. In budding yeast, Spt6 is a large 168 kilodalton (kD) protein containing 1451 amino acids (Swanson et al., 1990). An alignment of Spt6 amino acid sequences from *S. cerevisiae, S. pombe, C. elegans, D. melanogaster, Danio rerio,* and *Homo sapiens* has been analyzed for residues 298 through 1440 (Close et al., 2011). Of these residues, 42% are conserved, with 10% being invariant (Close et al., 2011). *S. cerevisiae* Spt6 has been predicted to contain several domains based on sequence analysis and structural information: an N-terminal domain, a helix-turn-helix domain, a

YqgF homology domain, a helix-hairpin-helix domain, a death-like domain, an S1 domain and tandem SH2 domains (Figure 1-2A) (Close et al., 2011; Dengl et al., 2009; Johnson et al., 2008).

The central core of Spt6 (indicated by the dashed box in Figure 1-2A) has approximately 25% pairwise sequence identity with the Tex bacterial transcription factor, which is involved in toxin expression in *Pseudomonas aeruginosa* (Johnson et al., 2008). This similarity to a bacterial transcription factor suggests that the central region of Spt6 is not involved in nucleosome-related functions and represents a more ancestral protein scaffold conserved from prokaryotes to eukaryotes (Close et al., 2011; Dengl et al., 2009; Johnson et al., 2008). The *P. aeruginosa* Tex protein structure has been solved at 2.3 Å resolution and revealed an elongated helical protein made up of putative nucleic acid binding domains (Johnson et al., 2008). This prokaryotic structure offered some initial insight into a possible structure for Spt6 (Johnson et al., 2008).

More recently, a nearly complete structure of *S. cerevisiae* Spt6 was solved using combined data from three independently determined partial structures crystallized at 2.7-3.3 Å resolution (Figure 1-2B) (Close et al., 2011). Similar to Tex, this structure indicates that *S. cerevisiae* Spt6 is mostly helical and organized around a single 80 Å helix at the center of the protein (Close et al., 2011). The central core domains of Spt6 (the HtH, YqgF, HhH and S1 domains) generally resemble those of the Tex protein (Close et al., 2011). However, while the HtH, YqgF and HhH domains have similar structures and locations in both Spt6 and Tex, the Spt6 S1 domain is rotated 80° and moved approximately 30 Å in relation to its Tex counterpart (Close et al., 2011; Johnson et al., 2008). In addition, while both Spt6 and Tex bind DNA, Tex requires the S1 domain for this activity while Spt6 does

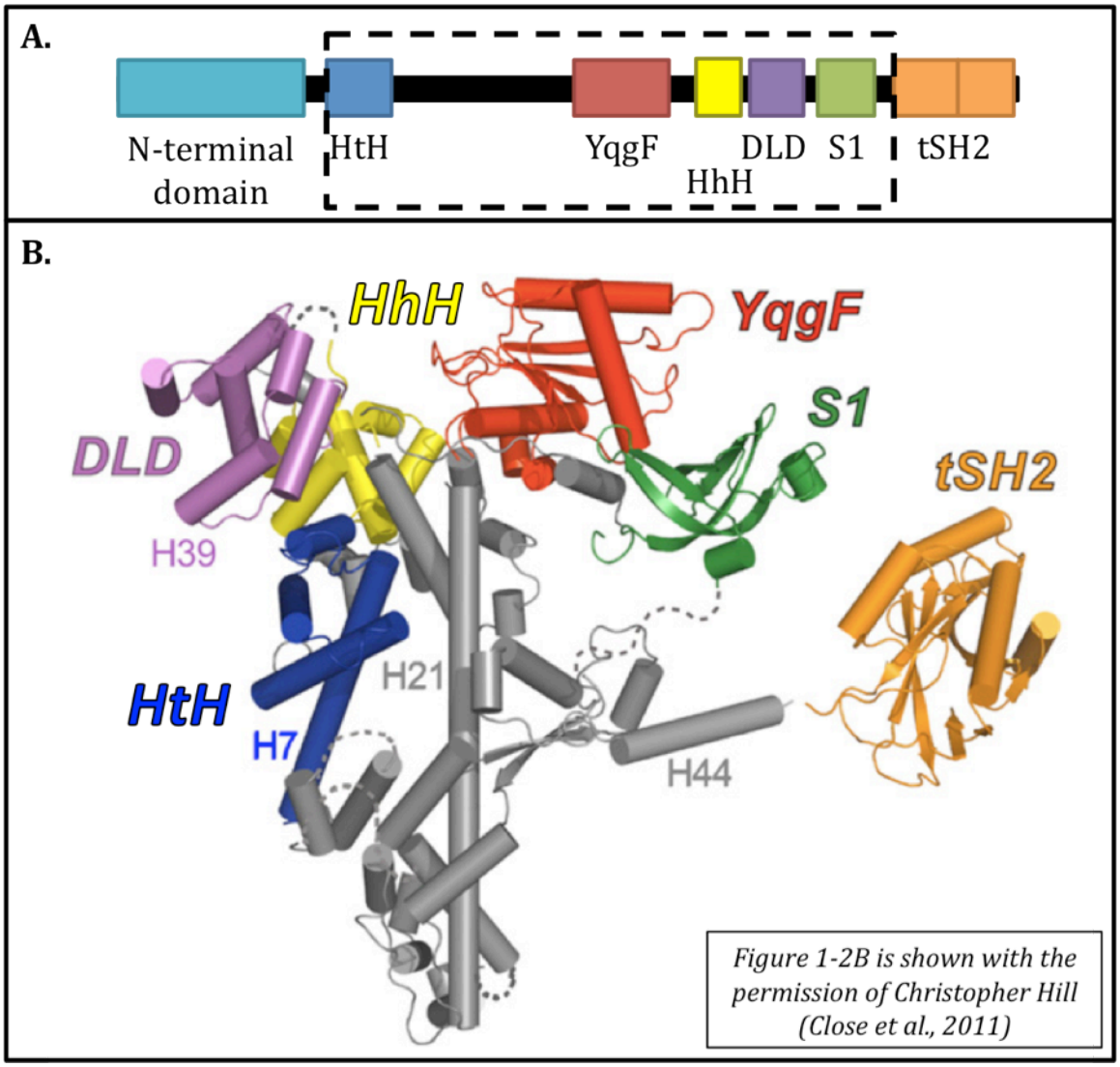


Figure 1-2: Domains of Spt6. A. Diagram of Spt6 and its known domains. *S. cerevisiae* Spt6 is 1451 amino acids long. The dashed box indicates the central core of Spt6 which has approximately 25% pairwise sequence identity with the *P. aeroginosa* Tex transcription factor. **B.** Model of the crystal structure of Spt6 (amino acids 298-1451), colored by domain according to the diagram in Part A above it. The N-terminal domain unstructured region is not shown. The Spt6 crystal structure is shown with the permission of Christopher Hill. (Close et al., 2011; Johnson et al., 2008)

not (discussed below) (Close et al., 2011; Johnson et al., 2008). In general, the *S. cerevisiae* Spt6 central core structure resembles its prokaryotic homologue in *P. aeruginosa* with the exception of alterations in the S1 domain.

Drawing from these studies and other work on the Spt6 C-terminal region (Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010), I summarize below the current knowledge of the domain structure of Spt6 (Table 1-2). It should be noted that although most Spt6 domains were defined based on structural or sequence similarity to previously characterized domains, most Spt6 domains are incompatible with their predicted function because of steric clash with predicted binding partners or lack of required catalytic residues (Close et al., 2011).

N-terminal Domain

The N-terminal domain (NTD) of Spt6 is comprised of the first 297 amino acids of Spt6, is highly acidic with a predicted pI of 4.3, and is believed to be disordered in the absence of binding partners (Figure 1-2A, light blue; not shown in Figure 1-2B) (Close et al., 2011). Due to the disordered nature of this region, the NTD structure has not been solved and is not shown in Figure 1-2B. As discussed in Chapter 2, residues 229-269 of the Spt6 NTD are required for binding to the transcription factor Spn1 and to nucleosomes in a mutually exclusive fashion (Diebold et al., 2010a; McDonald et al., 2010). Deletion of the region encoding the first 205 amino acids of the Spt6 NTD is lethal, while deletion of the region encoding the first 122 amino acids is synthetically lethal when combined with deletions of genes encoding components of the Paf1 transcription factor complex (Kaplan et al., 2005). It should be noted that *spt6* mutants encoding a deletion of the HhH domain

Table 1-2: Function of domains of Spt6. Summary of current knowledge of the functions of the domains of Spt6. (Close et al., 2011)

Domain	Predicted function	Experimentally-determined function
NTD	Binds histones	Binds Spn1 and nucleosomes
HtH	Binds dsDNA	Unknown
YqgF	Resolvase or RNase	Unknown – lacks residues for catalytic activity
HhH	Binds dsDNA or protein	Unknown
DLD	Activates apoptotic complexes	Unknown
S1	Binds nucleic acids	Unknown – not required for dsDNA binding
tSH2	Binds phosphotyrosine	Binds phosphorylated CTD of RNAPII

or the last C-terminal 170 amino acids are also lethal when combined with deletions of genes encoding Paf1 complex components (Kaplan et al., 2005). Altogether, this suggests a model whereby the Spt6 NTD and its interaction with Spn1 is important for regulating nucelosome binding and Spn1 may act as a switch for nucleosome disengagement. The effects of mutations in the Spt6 NTD are discussed extensively in Chapter 2.

Helix-turn-helix Domain

The Helix-turn-helix (HtH) domain of Spt6 (residues 336-442; Figure 1-2A, dark blue) was first identified based on structural information from the Spt6 homologue Tex in *P. aeruginosa* (Johnson et al., 2008). This domain was also observed in the recent *S. cerevisiae* structure (Figure 1-2B, dark blue) (Close et al., 2011). HtH domains generally bind DNA and are found in transcription factors (for example, sigma factors and c-Myb) (Aravind et al., 2005). However, unlike typical HtH domains, the *S. cerevisiae* Spt6 HtH domain structure is not compatible with DNA binding due to the steric clash that would result between the bound DNA molecule and other portions of the Spt6 protein structure (Close et al., 2011). Therefore, the function of this domain is still unclear. In Chapter 2 of this dissertation, I describe a mutation in this domain that causes decreased Spt6 protein levels.

YqgF Homologous Domain

This domain (residues 735-887; Figure 1-2A and B, red) was identified based on its sequence similarity to the *E. coli* YqgF protein, RuvC Holliday junction resolvases, and other RNase H-fold nuclease domains (Ponting, 2002). This class of domains is typically found in

nucleases involved in Holliday junction resolution and DNA recombination (Aravind et al., 2000). YqgF or YqgF-like domains are also found in 3' to 5' exonucleases, retroviral integrases, and DNA transposon integrases (Aravind et al., 2000). However, the YqgF homologous domain of Spt6 lacks the catalytic residues necessary for nuclease activity (Close et al., 2011). Therefore, it is unlikely to act as a nuclease and its function is unique from that of other YqgF domains that have nuclease activity.

Helix-hairpin-helix Domain

Helix-hairpin-helix (HhH) domains are typically involved in binding double-stranded DNA (dsDNA) although they can also mediate protein-protein interactions (Shao and Grishin, 2000). The Spt6 HhH domain(residues 933-1002; Figure 1-2A and B, yellow) was identified based on sequence homology (Doherty et al., 1996). Recent work suggests that this domain in Spt6 may actually contain two tandem HhH domains (Close et al., 2011). Interestingly, a region of Spt6 (residues 298-325), immediately adjacent to the NTD described above, wraps around the HhH domain and may prevent the HhH domain from binding dsDNA in the typical manner (Close et al., 2011). The exact function of the HhH domain in Spt6 is yet to be determined. Deletion of the region encoding the HhH domain (as in the *spt6-1004* mutant discussed in Chapters 1, 2, and 3) results in a number of mutant phenotypes including temperature-sensitivity, cryptic initiation, and an Spt$^-$ phenotype (Cheung et al., 2008; Kaplan et al., 2003).

Death-like Domain

One of the most mysterious domains of Spt6 is the most recently identified domain (residues 1019-1104; Figure 1-2A and B, purple), which is similar to a death domain (Close et al., 2011). Death domains are generally important for assembling and activating apoptotic and inflammatory complexes (Park et al., 2007). The purpose of this death-like domain (DLD) in a histone chaperone such as Spt6 remains to be discovered. Interestingly, the DLD domain is the most highly conserved region of the Spt6 protein surface and therefore is presumed to be important for interacting with other molecules (Close et al., 2011).

S1 Domain

The S1 domain (residues 1129-1219; Figure 1-2A and B, green) was first identified based on sequence homology (Ponting, 2002). S1 domains typically bind nucleic acids (Theobald et al., 2003). However, the S1 domain in yeast Spt6 is actually not required for dsDNA binding and it lacks the typical S1 nucleic acid binding residues (Close et al., 2011). Therefore, the function of the S1 domain in Spt6 is uncertain.

Tandem SH2 Domains

Src homology 2 (SH2) domains are very common in metazoans and generally recognize phosphorylated tyrosine residues in signal transduction cascades (Pawson, 2004). Interestingly, the only yeast protein known to contain an SH2 domain is Spt6 (Maclennan and Shaw, 1993). This has led to speculation that the Spt6 SH2 domain is a particularly unique or ancestral SH2 domain (Dengl et al., 2009).

As discussed in Chapter 2, a number of recent structural studies have shown that there are in fact not one but two SH2 domains (residues 1250-1440; Figure 1-2A and B, orange) in tandem at the C-terminus of Spt6 (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). The N-terminal SH2 domain has a typical SH2 domain sequence and structure while the C-terminal SH2 domain is less typical (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). Although its overall secondary structure is that of a standard SH2 domain, the C-terminal SH2 domain lacks sequence conservation with other SH2 domains and has an unusually shallow binding pocket with the critical binding residues missing (Close et al., 2011). The Spt6 tandem SH2 domains are attached to the Spt6 central core region by a flexible linker that may allow for more flexibility in interacting with binding partners (Close et al., 2011).

The tandem Spt6 SH2 domains are required for binding the phosphorylated CTD of RNAPII (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). Specifically, the SH2 domains bind T1p, S2p, S5p, and S7p phosphorylated RNAPII CTD, with a particularly strong affinity for T1p (Diebold et al., 2010b; Liu et al., 2011; Mayer et al., 2012; Sun et al., 2010). Mutations that change the proposed binding residues within the SH2 domains decrease binding with the RNAPII CTD partially but not completely, suggesting that each residue only partly contributes to RNAPII CTD binding or that not all key binding residues have been identified yet (Close et al., 2011; Diebold et al., 2010b). More detailed phenotypic analysis of SH2 domain deletions is described in Chapter 2.

Nucleic Acid Binding Capability

Previous work with the Tex homologue of Spt6 demonstrated that Tex binds nucleic acids (ssRNA, dsRNA, ssDNA, and dsDNA) *in vitro* with a strong preference for ssRNA (Johnson et al., 2008). The S1 domain was required for this nucleic acid binding (Johnson et al., 2008). However, in *S. cerevisiae* Spt6, the story is quite different. The central core of Spt6 (containing the HtH, YqgF, HhH, and DLD domains) binds dsDNA and the S1 domain is not required for this activity (Close et al., 2011). Future studies are needed to determine whether yeast Spt6 binds RNA and to determine which central core domain is responsible for dsDNA binding. Surprisingly, a shorter Spt6 N-terminal truncation construct (residues 315-1451) bound dsDNA with a higher affinity than full-length Spt6 (Close et al., 2011). This suggests that the N-terminus of Spt6 diminishes dsDNA binding, perhaps through interaction with nucleosomes or Spn1.

III. Spn1/Iws1

Discovery of Spn1

SPN1 (**S**uppresses **P**ostrecruitment functions gene **N**umber 1) was originally isolated from a screen for suppressors of a specific defect in TATA-Binding Protein (TBP) that impaired a post-recruitment defect in transcription activation (Fischbeck et al., 2002). In all organisms other than *S. cerevisiae*, Spn1 is known as Iws1 (**I**nteracts **W**ith **S**pt6; (Krogan et al., 2002)); it will be referred to as Spn1 throughout this dissertation. Mutations in *SPN1* also alter RNAPII transcription at some but not all inducible genes, increasing *CYC1* transcription under partially repressing and activating conditions and decreasing *HIS3*

transcription under inducing conditions (no effects were seen at *GAL7* or *SSA4*) (Fischbeck et al., 2002). In addition, *spn1* mutants have minimal effects on a subset of constitutively expressed genes including *HTA2, RPS4,* and *ENO2* (Fischbeck et al., 2002). Overall, these findings suggest that *SPN1* is important for transcriptional regulation of a subset of genes. *SPN1* was subsequently shown to encode a 46 kD protein essential for viability in budding yeast and conserved among eukaryotes (Fischbeck et al., 2002; Zhang et al., 2008). Interestingly, *SPN1* is not essential for viability in *S. pombe* (Kiely et al., 2011).

Biological Roles of Spn1

Spn1 physically interacts with Spt6, Spt5, and RNAPII, and *spn1* mutations confer an Spt$^-$ mutant phenotype (Fischbeck et al., 2002; Lindstrom et al., 2003; Zhang et al., 2008). As discussed in Chapter 2, Spn1 was found to bind to Spt6 within a section of the N-terminal domain of Spt6 that also binds histones in a mutually exclusive fashion (Diebold et al., 2010a; McDonald et al., 2010). Spn1 appears to block Spt6-nucleosome complex formation directly through covering the required binding site on Spt6 (McDonald et al., 2010). Interestingly, Spt6 and Spn1 have different genomic recruitment profiles even though they are both associated with elongating RNAPII (Mayer et al., 2010).

Although their functions do not completely overlap, Spn1 is involved in many similar processes to Spt6, including histone modification, transcription regulation, and mRNA processing and export (Fischbeck et al., 2002; Krogan et al., 2002; Lindstrom et al., 2003; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Interestingly, Spn1 binds Spt6 in some but not all contexts (Lindstrom et al., 2003; Yoh et al., 2007; Zhang et al., 2008). For example, Spt6 is associated with three different Spt5-RNAPII complexes while Spn1 co-

immunopurifies with only two of these (Lindstrom et al., 2003). These three RNAPII complexes include 1) hypophosphorylated $RNAPII_A$, 2) hyperphosphorylated $RNAPII_O$ with Abd1 (the cap methyltransferase) and a number of capping enzymes bound, and 3) hyperphosphorylated $RNAPII_O$ binding Cdc68/Pob3 but no longer associated with Spn1, Abd1 or other capping enzymes (Lindstrom et al., 2003). In addition, microarray data indicates that *spn1* and *spt6* mutants affect overlapping but distinct sets of genes, suggesting that Spn1 and Spt6 may have some distinct roles (Burckin et al., 2005).

Spn1 has been shown to interact with a wide array of other transcription factors and transcription-associated proteins. For example, human Spn1/IWS1 has been implicated in binding the transcription elongation factor TFIIS *in vitro* and may be responsible for TFIIS nuclear localization (Ling et al., 2006). Spn1 also binds PRMT5, the protein arginine methyltransferase which methylates the transcription elongation factor Spt5, regulating the interaction of Spt5 with RNAPII (Liu et al., 2007). In addition, Spn1 is involved in *Arabidopsis thaliana* plant steroid-induced gene expression, partially through interaction with the BES1 transcription factor that recruits Spn1 to promoters and transcribed regions of target genes under induced conditions (Li et al., 2010). These findings illustrate that Spn1 is a crucial transcription factor in humans and *Arabidopsis* as well as yeast, and appears to coordinate a number of important transcription factors including TFIIS.

Additional experiments raise the possibility that Spn1 may also have a role in repressing cryptic initiation. Human Spn1/IWS1 recruits HYPG/Setd2 histone methyltransferase to actively transcribing RNAPII and is required for H3K36 trimethylation across *c-Myc, HIV-1,* and *PABPC1* ORFs (Yoh et al., 2008). In yeast, H3K36

trimethylation recruits Rpd3-type histone deacetylases that reestablish hypoacetylated reassembled chromatin and prevent cryptic and other intragenic transcription (Carrozza et al., 2005; Keogh et al., 2005; Lee and Shilatifard, 2007). Depletion of human Spn1/IWS1 *in vivo* also increases H3K27 trimethylation at the promoter proximal region of the human *PABPC1* gene and increases histone H4 acetylation over *HIV-1* and *PABPC1* ORFS and promoters (Yoh et al., 2008). Therefore, it is possible that Spn1 has a role in cryptic initiation that has not yet been defined.

Although there is no truly comprehensive model tying together the numerous functions of Spn1 in gene expression, one model of Spn1 function in transcription initiation has been proposed based on studies of its function at the *CYC1* gene. At *CYC1,* RNAPII recruits Spn1 and the current model is that Spn1 negatively regulates RNAPII transcription initiation by preventing binding of the chromatin remodeler Swi/Snf (Zhang et al., 2008). The subsequent interaction of Spn1 with Spt6 allows binding of Swi/Snf which correlates with induced levels of gene expression, suggesting that the Spt6-Spn1 interaction is important for the switch from inactive to active transcription (Zhang et al., 2008). Interestingly, *CYC1* transcription is increased in microarray data sets for both *spn1* and *spt6* mutants (Burckin et al., 2005). Although not comprehensive, this model of action of Spn1 at the *CYC1* gene summarizes an aspect of Spn1 function in transcription initiation.

In addition to its roles in gene expression, Spn1 is important for mRNA processing and export. Spn1 is involved in mRNA splicing in *S. cerevisiae,* and *in vivo* knock-down of human Spn1/IWS1 leads to nuclear retention of poly(A)$^+$ RNAs and abnormal mRNA processing (Yoh et al., 2007; Yoh et al., 2008). In addition, human Spn1 binds and helps recruit the RNA export factor REF1/Aly to the *c-Myc* ORF during transcription (Yoh et al.,

2007; Yoh et al., 2008). Overall, Spn1 appears to have crucial roles in both transcription and transcription-related processes such as mRNA processing and export.

IV. Spt6, DNA Damage Repair, and Transcription-associated Mutagenesis

Brief Overview of DNA Damage and Double-strand Break Repair

Cells are constantly experiencing DNA damage and double-strand breaks (DSBs), whether from environmental or endogenous toxins or from physiological processes such as meiosis, yeast mating type switching, and V(D)J recombination (Gospodinov and Herceg, 2013). DNA damage is extremely disruptive to any organism and, if left unrepaired, can lead to mutations, cell cycle arrest, cell death, and cancer development (Gospodinov and Herceg, 2013; Kinoshita et al., 2009; Toh and Lowndes, 2003).

In *S. cerevisiae*, homologous recombination (HR) is the preferred method of repair for DSBs (Lisby and Rothstein, 2009). In HR, DSB detection leads to recruitment and activation of the PI3K-like kinases Mec1 and Tel1, equivalents of mammalian ATR and ATM (Chen et al., 2007; Lisby et al., 2004; Lisby and Rothstein, 2009; Longhese et al., 2003; Smolka et al., 2007; Tsabar and Haber, 2013). With the help of the adaptor molecules Rad9 and Mrc1, Mec1 and Tel1 phosphorylate and activate the kinases Chk1 and Rad53 (Chen et al., 2007; Longhese et al., 2003; Smolka et al., 2007; Tsabar and Haber, 2013). These kinases, in turn, regulate cell cycle arrest, transcription of genes involved in DNA repair, replication fork stabilization, chromosome segregation delay, and repair of DNA (Smolka et al., 2007; Tsabar and Haber, 2013). In particular, Rad53 appears to act through phosphorylation of Dun1, a kinase that plays a role in cell cycle arrest (Chen et al., 2007).

Simultaneously, Rad52 is recruited to DSBs, forming distinct foci and recruiting many proteins directly involved in recombinational DNA repair such as Rad51 and Rad59 (Lisby et al., 2004; Lisby and Rothstein, 2009).

Chromatin Remodeling in DNA Damage Repair

In recent years, it has become clear that chromatin remodeling has a crucial role in the DNA damage repair (DDR) pathway (Gospodinov and Herceg, 2013; Seeber et al., 2013; Tsabar and Haber, 2013; Tsukuda et al., 2005). As in the case of transcription, chromatin structure also poses an obstacle to DDR, and there are many parallels between chromatin regulation in transcription and in DDR, with a number of the key players involved in both (Gospodinov and Herceg, 2013; Huertas et al., 2009; Seeber et al., 2013; Tsabar and Haber, 2013; Tsukuda et al., 2009). A number of studies indicate that changes in histone structure and modifications are required to recruit factors necessary for DDR (Groth et al., 2007; Tsukuda et al., 2009). A growing list of histone chaperones and chromatin remodeling complexes have been implicated in this process, including Arp4, Hif1, CAF-1, Asf1, DNMT1, Rad54, and the ISW1a, ISW1b, ISW2, CHD1, FUN30, INO80, SWR1, RSC, and Swi/Snf complexes (De Koning et al., 2007; Gospodinov and Herceg, 2013; Groth et al., 2007; Kim and Haber, 2009; Seeber et al., 2013; Tsabar and Haber, 2013). Their roles in DDR include 1) promoting phosphorylation and Rad51 and Mre11 recruitment at damage sites, 2) chromatin relaxation and histone mobilization, 3) binding and propagating phosphorylated histone H2A (equivalent to γH2A.X in vertebrates), 4) direct roles in DNA end resection and priming breaks for repair, 5) maintaining the G2/M checkpoint and DNA damage

checkpoint, and 6) promoting HR in general (Gospodinov and Herceg, 2013; Seeber et al., 2013; Tsabar and Haber, 2013).

The relationship between chromatin structure and DNA damage repair has significant biological and medical implications for tumor biology, where chromosomal instability and abnormal DDR are characteristic features of cancer cells (Gospodinov and Herceg, 2013; Stratton et al., 2009). Remarkably, nearly half of the 120 key tumor driver mutations identified are in genes encoding proteins directly involved in regulating chromatin through modifying histones and DNA (Gospodinov and Herceg, 2013; Suva et al., 2013; Vogelstein et al., 2013). In light of this, several chromatin remodelers have been suggested as possible therapeutic targets (Gospodinov and Herceg, 2013). Therefore, understanding the interplay between chromatin remodelers and DDR is imperative for advancing our knowledge of cancer biology, mutagenesis, and cell growth.

Possibility of a Role for Spt6 in DNA Damage Repair

Given the crucial role of Spt6 in transcription regulation and chromatin structure, an intriguing possibility is that Spt6 is involved in DNA damage that occurs during transcription, a phenomenon known as transcription-associated mutagenesis or TAM. Spt6 has been studied nearly exclusively in the context of transcription and chromatin, leaving any potential role in DNA damage mostly unexplored.

The initiative to investigate this question arose from an unexpected observation based on nucleosome occupancy data for chromosome III in an *spt6* mutant, *spt6-1004* (Ivanovska et al., 2011). In an *spt6-1004* mutant, nucleosomes are lost from highly transcribed genes, consistent with the previously proposed role of Spt6 in replacing

nucleosomes in the wake of transcription (Hartzog et al., 1998; Ivanovska et al., 2011; Kaplan et al., 2003). However, this nucleosome loss does not correlate with Spt6-mediated changes in mRNA levels or cryptic initiation. For example, there are genes with significant nucleosome loss but no change in transcription, and there are also genes with cryptic initiation but no appreciable nucleosome loss (Ivanovska et al., 2011). This suggests that the effect of Spt6 on chromatin regulation is independent of its effect on transcription. The intriguing unanswered question is why is it important for nucleosomes to be replaced over highly transcribed coding regions if transcription and cryptic initiation are not affected by nucleosome loss. One hypothesis is that the histone chaperone activity of Spt6 is important for preventing transcription-associated mutagenesis (TAM). This hypothesis is explored further in Chapter 3.

Transcription-associated Mutagenesis Overview

TAM refers to the observation that increased transcription of a gene leads to higher levels of mutagenesis across the gene (Lippert et al., 2011; Mischo et al., 2011; Takahashi et al., 2011). This phenomenon has been observed in bacteriophage T7, *E. coli*, *S. cerevisiae,* and mammalian cells (Beletskii and Bhagwat, 1996; Beletskii et al., 2000; Datta and Jinks-Robertson, 1995; Hendriks et al., 2010; Hendriks et al., 2008; Klapacz and Bhagwat, 2002). In yeast, induction of transcription with a strong promoter increases mutagenesis between 7- to 30-fold, depending on the promoter (Lippert et al., 2011; Takahashi et al., 2011). While TAM appears to increase most classes of mutations, elevations in 2-3 bp deletions are particularly characteristic (Lippert et al., 2011; Takahashi et al., 2011). The mechanism of TAM is unclear; however, one explanation is that open, transcriptionally-active

chromatin is more sensitive to DNA damage (Lippert et al., 2011; Svejstrup, 2010). This would fit well with the role of Spt6 as a histone chaperone, restoring chromatin following transcription and protecting from mutagenesis. Other possible mechanisms for TAM include 1) repair of stalled Topoisomerase I-DNA complexes, 2) collision between replication and transcription machineries leading to interference with DNA replication precision, 3) development of transient super-coils producing ssDNA which is more sensitive to mutation and can obstruct RNAPII, or 4) some combination of the above mechanisms (Lippert et al., 2011; Mischo et al., 2011; Svejstrup, 2010; Takahashi et al., 2011).

Evidence for a Role of Spt6 in DNA Damage Repair

The hypothesis that Spt6 is involved in some aspect of DDR is not novel. It had previously been shown that a truncated *spt6* allele which contains a stop codon at amino acid 1274 (*spt6-50*) is sensitive to hydroxyurea (Kaplan, 2002). Sensitivity to hydroxyurea, a ribonucleotide reductase inhibitor that depletes dNTP pools and causes DNA single-strand breaks, can indicate defects in DDR (Hampsey, 1997; Osterman Golkar et al., 2013; Walker et al., 1977). Subsequently, several *spt6* alleles were found to be sensitive to phleomycin, a radiomimetic that induces DSBs (I. Ivanovska, unpublished data; E. Loeliger, unpublished data). Additional work has indicated a synthetic-sick genetic interaction between *spt6-1004* and deletion of the gene encoding Dun1, a DNA damage checkpoint kinase involved in the main DSB repair pathway in *S. cerevisiae* (I. Ivanovska, unpublished data). These DDR-related phenotypes of *spt6* mutants suggest a link between Spt6 and DDR.

Role of Spt6 in DNA Recombination

In addition, *spt6* mutants have a hyper-recombination phenotype, indicating that Spt6 affects DNA recombination levels. In the *spt6-140* mutant, gene conversion and deletion events increase up to 15-fold across seven independent direct or inverted repeat reporters (Malagon and Aguilera, 1996). This *spt6* hyper-recombination phenotype is dependent on Rad52 and Rad1-Rad59 (Malagon and Aguilera, 2001). Northern blot and MNase digest analysis showed that the *spt6-140* mutant decreases transcription and alters chromatin (exposing different MNase hypersensitive sites) over the inverted repeat reporters used (Malagon and Aguilera, 2001). Interestingly, the *spt6* hyper-recombination phenotype was not present in the absence of transcription (Malagon and Aguilera, 2001). Overall, an *spt6* mutant causes a distinct hyper-recombination phenotype over several repeat reporters, most likely as a result of defects in transcription and chromatin over those regions.

V. Yeast as a Model Organism

All studies of Spt6 in this dissertation were performed in the budding yeast, *S. cerevisiae*. Budding yeast (also known as baker's yeast) has been closely entwined with human history for thousands of years, with the first evidence of wine and other fermented beverage production in Iran and Egypt in 6000 B.C. and 3000 B.C., respectively (Legras et al., 2007). In addition to its importance for diet staples such as bread, beer, and wine, yeast has played a crucial role in the development of modern day biology and genetics.

First used as a model organism approximately 75 years ago, *S. cerevisiae* is known as the "*E. coli* of the eukaryotes", combining the convenience of prokaryotic organisms and the complex biology of eukaryotic organisms regarding organelles, cell cycle, aspects of chromatin, and other processes (Griffiths, 2008). Yeast are small, hardy, and divide every 90 minutes making it possible to generate large numbers quickly and easily. They can also be grown in liquid medium in large batches or on solid agar medium, allowing screening for mutant phenotypes. In addition, many years of study have generated a wide variety of reagents, yeast mutants, and plasmids, making yeast experiments even more convenient. High levels of homologous recombination, the ability to exist stably as a haploid or diploid, and the ability to separate single haploid offspring through tetrad dissection makes yeast an ideal genetic model. In addition, cellular processes in yeast are largely conserved with those of their larger eukaryotic counterparts (for example, mice and humans) (Griffiths, 2008; Hahn and Young, 2011; Rando and Winston, 2012). Therefore, yeast is a powerful tool for experiments addressing complex biological questions that would not be feasible in other eukaryotic organisms.

Indeed, yeast has proven to be essential for exploring many areas of biology. Work with petite yeast colonies led to a better understanding of mitochondrial genetics which has given insight into human neurological and muscular illnesses such as mitochondrial myopathy (progressive muscle weakness) and myoclonus epilepsy (a form of epilepsy typified by progressive mental deterioration and muscle spasms) (Barnett, 2007). Likewise, the Nobel Prize-winning identification of key cell cycle regulators Cdc2/CDK1, cyclins, and the CDC genes by Sir Paul Nurse, Timothy Hunt, and Leland Hartwell using fission yeast, sea urchins, and budding yeast, respectively, forms the basis of human cancer

research today (Barnett, 2007; Evans et al., 1983; Hartwell et al., 1970; Hartwell et al., 1973; Lee and Nurse, 1987; Nurse and Thuriaux, 1980; Pines and Hunt, 1987).

The ground-breaking work in the transcription, chromatin and DNA damage repair fields was originally done in *S. cerevisiae*, making budding yeast a well-established system for studying these processes (Botstein and Fink, 2011; Hahn and Young, 2011; Rando and Winston, 2012; Toh and Lowndes, 2003). Also, much of transcription and DNA damage repair is conserved among eukaryotes (Lisby et al., 2004). Taken together, these facts made budding yeast the ideal organism for my dissertation research, allowing the study of complex eukaryotic biology.

VI. Overview of Dissertation

Spt6 has been shown to function in many aspects of gene expression, including nucleosome assembly, transcription initiation and elongation, and mRNA processing and export. Overall, despite many genetic and biochemical studies of Spt6 described in this introduction, the mechanism of Spt6 is still unclear. The goal of my dissertation has been to further characterize the role of Spt6 in chromatin structure, transcription, and DNA damage repair.

In Chapter 2, I have performed a structure-function analysis of Spt6 using three separate approaches. First, I employed a random insertion mutagenesis that has identified sixty-seven *spt6* mutants. While these mutants did not provide information regarding known domains, some have phenotypes that may prove useful for future study. Second, in a collaborative project with the Romier lab, I studied the functional role of the Spt6 SH2

domains. We have shown that deletion of the region of *SPT6* encoding the SH2 domains causes severe mutant phenotypes without affecting Spt6 protein levels, demonstrating the importance of the SH2 domains of Spt6. Third, in an additional project with the Romier lab, I showed that mutations in the region of Spt6 that interacts with the conserved transcription factor Spn1 impair Spt6 function *in vivo*. Overall, this multi-pronged structure-function analysis of Spt6 has provided new insights into the uses and limitations of insertion mutagenesis, the tandem SH2 domains of Spt6, and the Spt6-Spn1 interaction.

In Chapter 3, I explored the tantalizing hypothesis that Spt6 facilitates DNA damage repair during transcription. To address this possibility, I have attempted to test for a possible role for Spt6 in transcription-associated mutagenesis (TAM). After employing several types of *in vivo* assays, I conclude that a possible role for Spt6 in TAM is uncertain, as the results reproducibly vary depending on the assay used. Thus, understanding this aspect of Spt6 function awaits better assays and understanding of TAM.

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Chapter 2

Structure-Function Analysis of Spt6 Through Mutant Analysis

Attribution of Experiments in Chapter 2

All yeast strain construction and mutant analyses were preformed by Erin Loeliger. The experiments in Figures 9, 10, 11, and 12 were done by Marie-Laure Diebold and Christophe Romier. The construction of yEL165 was done with the assistance of Natalie Kuldell. The original pMG2 plasmid (MG4-1) was constructed by Mihir Gupta. All other experiments and figures were completed by Erin Loeliger.

Abstract

Spt6, a key factor involved in regulating chromatin structure, is conserved throughout eukaryotes. Spt6 has been shown to function in many aspects of gene expression, including nucleosome assembly, transcription initiation and elongation, and mRNA processing and export. In addition, Spt6 has several conserved domains; however, little is known about their functions. I have performed a structure-function analysis of Spt6 using three separate approaches. First, I employed a random insertion mutagenesis that has identified sixty-seven mutants. While these mutants did not provide information regarding known domains, some have phenotypes that may prove useful for future study. Second, in a collaborative project with the Romier lab, I studied the functional roles of the Spt6 SH2 domains. I have shown that deletion of the region of *SPT6* encoding the SH2 domains causes severe mutant phenotypes without affecting Spt6 protein levels, demonstrating the importance of the SH2 domains of Spt6. Third, in an additional collaboration with the Romier lab, I showed that mutations that alter the region of Spt6 that interacts with the conserved transcription factor Spn1 impair Spt6 functions *in vivo*. Overall, this multi-pronged structure-function analysis of Spt6 has provided new insights into the uses and limitations of insertion mutagenesis, the tandem SH2 domains of Spt6, and the Spt6-Spn1 interaction.

Introduction

For large multi-domain proteins, elucidating the function of each domain is often the key to the puzzle of understanding the function of the overall protein. In the case of Spt6, it has been shown to have a number of different putative domains including an N-terminal domain, a helix-turn-helix domain, YqgF homology domain, a helix-hairpin-helix domain, a death-like domain, an S1 domain and a tandem SH2 domain (see Chapter 1 for more details) (Close et al., 2011; Diebold et al., 2010b; Johnson et al., 2008; Liu et al., 2011; Maclennan and Shaw, 1993; Sun et al., 2010). The phenotypes of yeast *spt6* mutants suggest that Spt6 has numerous roles *in vivo* and interacts with a number of other proteins (Adkins and Tyler, 2006; Ardehali et al., 2009; Bucheli and Buratowski, 2005; Carrozza et al., 2005; Chen et al., 2012; Cheung et al., 2008; Chu et al., 2006; Close et al., 2011; Degennaro et al., 2013; Diebold et al., 2010a; Diebold et al., 2010b; Endoh et al., 2004; Formosa et al., 2002; Hartzog et al., 1998; Ivanovska et al., 2011; Jensen et al., 2008; Kaplan et al., 2005; Kaplan et al., 2003; Kiely et al., 2011; Liu et al., 2011; McDonald et al., 2010; Sun et al., 2010; Wang et al., 2013; Winkler et al., 2000; Yoh et al., 2007; Yoh et al., 2008; Youdell et al., 2008).

Spt6 is generally thought to be a histone chaperone which acts by restoring histones to chromatin following transcription by RNA polymerase II (RNAPII) (Ardehali et al., 2009; Svejstrup, 2003). However, the mechanisms of action of Spt6 are still unclear. In addition, the functions of most domains within Spt6 have yet to be determined.

To address these questions, I employed a three-pronged approach for structure-function analysis of Spt6. The first approach involved an unbiased random insertion

mutagenesis of the *SPT6* gene. The advantage of this transposon-based insertion mutagenesis is that it produces single mutations that are easy to locate within the target gene (Bachman et al., 2002; Biery et al., 2000a; Biery et al., 2000b; Milutinovich et al., 2007). In addition, this protocol is highly efficient, generating a large number of mutants quickly. Because the mutagenesis is performed *in vitro*, this method is unbiased and random, avoiding interference from chromatin and other cellular processes. Most importantly, insertion mutagenesis has been used successfully for structure-function analysis by other groups (Bachman et al., 2002; Biery et al., 2000a; Biery et al., 2000b; Milutinovich et al., 2007).

The second approach for structure-function analysis focused on the C-terminal domain of Spt6, comprised of two SH2 domains referred to here as $SH2_1$ and $SH2_2$. The well-established $SH2_1$ domain (residues 1250-1353) of Spt6 is believed to play a critical role in Spt6 function (Close et al., 2011; Dengl et al., 2009; Endoh et al., 2004; Liu et al., 2011; Maclennan and Shaw, 1993; Yoh et al., 2007). At the time of the start of my dissertation work, it was the best-studied domain of Spt6 and, in humans, experiments had shown it helped mediate the interaction of Spt6 with RNAPII (Endoh et al., 2004; Yoh et al., 2007). Deletion of the sequence encoding the tandem SH2 domains ($SH2_1$ and $SH2_2$) in *S. cerevisiae* leads to significantly decreased cell growth rate and changes in mRNA levels of 204 genes (Dengl et al., 2009). Interestingly, until very recently, the $SH2_1$ domain was the only known SH2 domain in the *S. cerevisiae* genome (Dengl et al., 2009; Maclennan and Shaw, 1993). However, more recent structural analysis of Spt6 (discussed in this chapter) identified a second adjacent SH2 domain (residues 1354-1440, referred to here as $SH2_2$) that was not originally recognized by sequence homology analysis (Close et al., 2011;

Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). Yet the biological significance of this novel SH2 domain was unexplored *in vivo*, and I set out to investigate this question.

The third approach to Spt6 structure-function analysis focused on the binding interaction between Spt6 and its conserved binding partner, Spn1. Like Spt6, Spn1 is generally essential for viability and is conserved throughout eukaryotes (Fischbeck et al., 2002; Zhang et al., 2008). Previous work indicates that Spn1 physically interacts with Spt6 and regulates recruitment of Spt6 during transcription initiation (Fischbeck et al., 2002; Krogan et al., 2002; Lindstrom et al., 2003; Yoh et al., 2007; Zhang et al., 2008). Spn1 appears to be crucial for a number of Spt6 functions including mRNA processing, chromatin regulation, and the transition from inactive to actively transcribing RNAPII at certain promoters (McDonald et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). However, little is known about whether Spn1 is universally required for Spt6 recruitment and function.

Preliminary data at the time from our collaborators had shown that the N-terminal domain (NTD) of Spt6 is essential for interaction with Spn1 in the eukaryotic parasite *Encephalitozoon cuniculi* (Diebold et al., 2010a). This model organism was used initially to make structural studies possible (see Materials and Methods for more details). Alignment of the Spt6 NTD sequences from various organisms suggested an evolutionarily conserved region from residues 245 to 261 in *S. cerevisiae,* and the *S. cerevisiae* residues 229-269 were shown to be necessary and sufficient for Spn1 binding. Using Spn1-Spt6 NTD structural data as well as targeted mutation of conserved residues identified from the alignment, our collaborators constructed three *spt6* mutants likely to affect Spn1 binding. When *E. cuniculi* versions of these three *spt6* mutants were examined *in vitro*, all three

mutants caused loss of the *E. cuniculi* Spt6-Spn1 interaction. However, these mutants had previously only been examined in an *in vitro E. cuniculi* system. I sought to examine these mutants in an *in vivo* yeast system.

Overall, the multi-pronged structure-function analysis of Spt6 described in this chapter has proved useful for testing the function of specific Spt6 domains. In addition to clarifying the roll of the tandem SH2 domains, our work has also helped illuminate the binding site between Spt6 and Spn1. I have also generated a wide variety of new Spt6 insertion mutants, as well as C-terminal truncations and Spn1-related point mutants, which can be used by future scientists.

Materials and Methods

Tn7 Insertion Mutagenesis

Tn7 insertion mutagenesis was performed as previously described (Bachman et al., 2002; Biery et al., 2000a; Biery et al., 2000b; Milutinovich et al., 2007) using the GPS™-LS Linker Scanning System (New England Biolabs; Figure 2-1). The insertions were located by *Pme*I restriction digestion and sequencing. The target for mutagenesis was pMG2 (Table 2-1), a *CEN* plasmid that contains a construct encoding C-terminally MYC-tagged Spt6 driven by the *GAL1* promoter. This promoter causes *SPT6* to be over-expressed in the presence of galactose. Experiments were performed (data not shown) to demonstrate that the MYC tag has no effect on Spt6 function.

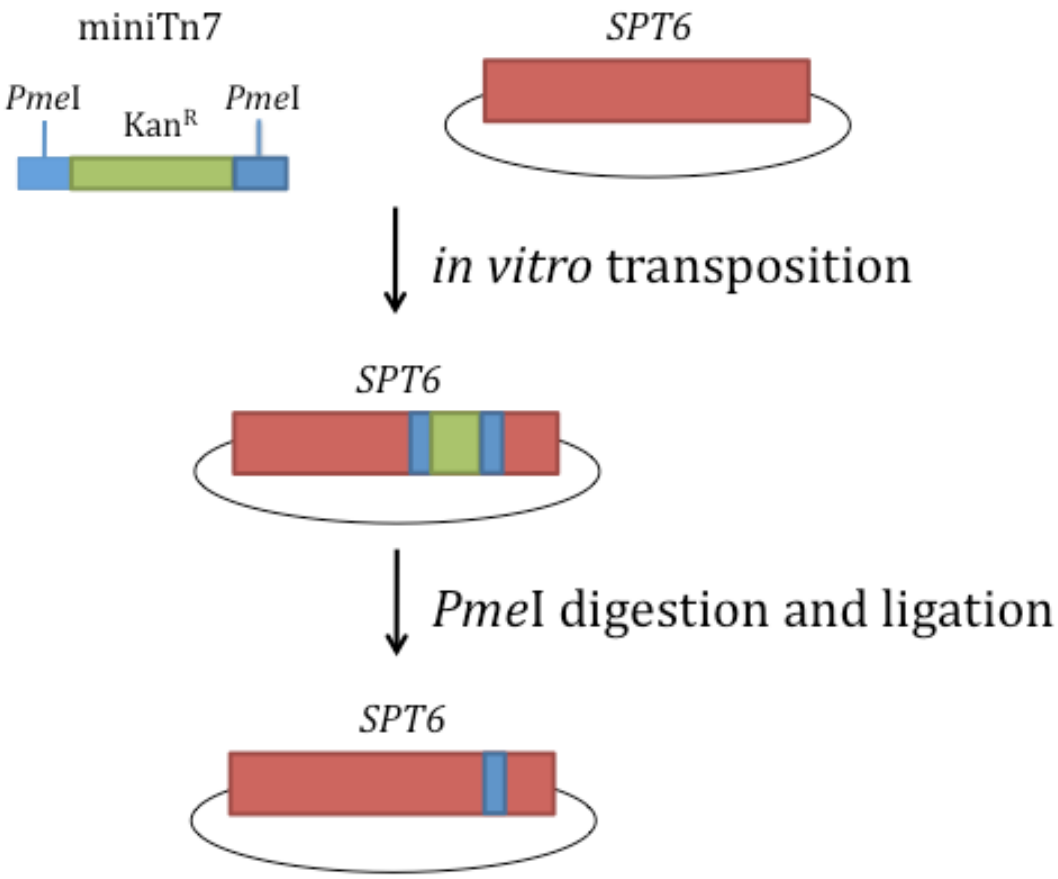

Figure 2-1: Overview of transposon mutagenesis. Mutagenesis is accomplished by random insertion of a derivative of the bacterial Tn7 transposon (denoted here as miniTn7) (Biery et al, 2000b) into the target DNA of interest (red), followed by digestion to remove the majority of the miniTn7 element. The miniTn7 construct contains a Kanamycin-resistant cassette (green) flanked by *Pme*I digest sites (blue). Following the *in vitro* transposition reaction, a library of plasmids is generated, each with a single insertion. Subsequent *Pme*I digestion and ligation removes the majority of the miniTn7 element, leaving a 15 base pair sequence containing a unique *Pme*I digest site (blue). (This figure is adapted from the New England Biolabs instruction manual; Biery et al, 2000b)

Table 2-1: Plasmids Used In This Chapter.

Plasmid	Purpose	Genotype
pCC11 (FB1053)	Strain construction	YCp50 backbone *AmpR CEN ARS URA3 SPT6⁺*
pCK134 (FB2338)	Control for spot tests	pRS414 backbone *FLAG-spt6-50* (Truncation mutant: K1274Stop mutation) *TRP1 CEN ARS*
pEL183 (FB2699)	Template for Quikchange Mutagenesis	pRS414 backbone *FLAG-SPT6 TRP1 CEN ARS*
pEL187 (ELB187)	*spt6-GG* (Spn1-binding site mutant)	pRS414 backbone *FLAG-spt6-GG* (G250A, G252A) *TRP1 CEN ARS*
pEL190 (ELB190)	*spt6-YW* (Spn1-binding site mutant)	pRS414 backbone *FLAG-spt6-YW* (Y255A, W257A) *TRP1 CEN ARS*
pEL193 (ELB193)	*spt6-IF* (Spn1-binding site mutant)	pRS414 backbone *FLAG-spt6-IF* (I248A, F249A) *TRP1 CEN ARS*
pEL203 (ELB203)	*spt6-Y* (Spn1-binding site mutant)	pRS414 backbone *FLAG-spt6-Y* (Y255A) *TRP1 CEN ARS*
pEL207 (ELB207)	*spt6-W* (Spn1-binding site mutant)	pRS414 backbone *FLAG-spt6-W* (W257A) *TRP1 CEN ARS*
pFA6a-C-TAP4 -natMX6 (FB2520)	SH2 truncation mutant strain construction	pFA6a backbone C-TAP4-NatMX6
pGP26 (FB1167)	Control for spot tests	YCp50 backbone *AmpR CEN ARS URA3 spt6-140*
pMG2 (MG4-1)	Template for Tn7 insertion mutagenesis	pBY011 backbone *AmpR CEN ARS LEU2 URA3 pGAL1-SPT6-13xMyc*
spt6::Tn7-X	Tn7 insertion mutant library	Same as pMG2 above with a Tn7 insertion at location X within *SPT6*

Quikchange Mutagenesis and Plasmid Transformation

Mutations encoding the amino acid changes I248A/F249A, G250A/G252A, Y255A/W257A, Y255A, and W257A in *S. cerevisiae SPT6* were each individually introduced into a copy of *SPT6* on a pRS414-based (*TRP1 CEN*) plasmid (pEL183) (Table 2-1). Mutagenesis was performed using the Quikchange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) and primers listed in Table 2-2. The resulting plasmids (pEL187, pEL190, pEL193, pEL203, and pEL207) were used to transform the *S. cerevisiae* strain FY857 (Table 2-3) using standard protocols (Ausubel et al., 1987). A 5-fluoroorotic acid (5-FOA) plasmid shuffle was performed to obtain a strain that contained only the plasmid with the *spt6* mutant allele (Boeke et al., 1984).

Yeast Strain Construction

All yeast strains (Table 2-3) were constructed by standard methods (Ausubel et al., 1987). The Tn7-based insertion mutant candidates (denoted *spt6::Tn7*) on plasmids were screened for phenotypes after transformation into yeast strain yEL22, which contains an *SPT6* deletion allele (*spt6Δ::LEU2*) in the genome and a *TRP1*-marked wild-type *SPT6 CEN* plasmid (pEL183). Transformants were screened for recessive phenotypes after loss of the wild-type *SPT6* plasmid (Figure 2-2A), detected by using 5-fluoroanthranilic acid (5-FAA) (Toyn et al., 2000), and for dominant phenotypes in the presence of the wild-type *SPT6* plasmid (Figure 2-2B).

For *spt6::Tn7* mutant integration into the genomic *SPT6* locus, a two-step yeast transformation was performed using the diploid strain yEL165. This diploid strain contains a DNA construct encoding FLAG-tagged *SPT6* with a *NatMx* cassette

Table 2-2: Primers Used In This Chapter.

Primer	Purpose	Sequence (5' to 3')
ELO11	Use with ELO12 and Quikchange Mutagenesis Kit to change Spt6 residues Ile248 and Phe249 to alanines - 5'	ggataagattgacgagatgtatgacgct gctggtgatggtcatgactacgattgg
ELO12	Use with ELO11 and Quikchange Mutagenesis Kit to change Spt6 residues Ile248 and Phe249 to alanines - 3'	ccaatcgtagtcatgaccatcaccagca gcgtcatacatctcgtcaatcttatcc
ELO13	Use with ELO14 and Quikchange Mutagenesis Kit to change Spt6 residues Gly250 and Gly252 to alanines - 5'	agatgtatgacatttttgctgatgctca tgactacgattgggc
ELO14	Use with ELO13 and Quikchange Mutagenesis Kit to change Spt6 residues Gly250 and Gly252 to alanines - 3'	gcccaatcgtagtcatgagcatcagca aaaatgtcatacatct
ELO15	Use with ELO16 and Quikchange Mutagenesis Kit to change Spt6 residues Tyr255 and Trp257 to alanines - 5'	gacgagatgtatgacatttttggtgatggtcatgac gctgatgctgctttagaaattgaaaatgaagaactaga aaatggt
ELO16	Use with ELO15 and Quikchange Mutagenesis Kit to change Spt6 residues Tyr255 and Trp257 to alanines - 3'	accattttctagttcttcattttcaatttctaaagcagca tcagcgtcatgaccatcaccaaaaatgtcatacatctcgtc
ELO33	Use with FO6852 to insert *TAP-NatMX* cassette into *SPT6* after codon 1250 (bp 3750) to generate *spt6-ΔTandem::TAP* - 5'	gaggaaattgatgatggcagaagcccgtgcaaagagaa cacggatccccgggttaattaa

Table 2-2: Primers Used In This Chapter (Continued).

Primer	Purpose	Sequence (5' to 3')
ELO34	Use with FO6852 to insert *TAP-NatMX* cassette into *SPT6* after codon 1351 (bp 4050) to generate *spt6-ΔSH2₂::TAP* - 5'	agaatatcttcaaaacaaggtaaggctcttgaatgaaa tgcggatccccgggttaattaa
ELO43	Use with ELO44 and Quikchange Mutagenesis Kit to change Spt6 residue Tyr255 to alanine - 5'	gtatgacatttttggtgatggtcatgacgctgattgggc tttagaaattgaaaatgaag
ELO44	Use with ELO43 and Quikchange Mutagenesis Kit to change Spt6 residue Tyr255 to alanine - 3'	cttcattttcaatttctaaagcccaatcagcgtcatga ccatcaccaaaaatgtcatac
ELO45	Use with ELO46 and Quikchange Mutagenesis Kit to change Spt6 residue Trp257 to alanine - 5'	gacatttttggtgatggtcatgactacgatgctgcttt agaaattgaaaatgaagaac
ELO46	Use with ELO45 and Quikchange Mutagenesis Kit to change Spt6 residue Trp257 to alanine - 3'	gttcttcattttcaatttctaaagcagcatcgtagtcatga ccatcaccaaaaatgtc
ELO103	Use with ELO105 to amplify region surrounding *spt6-543* Tn7 insertion (for Tn7 mutant integration into genome) - 5'	agattcgaagctggtcccta
ELO105	Use with ELO103 to amplify region surrounding *spt6-543* Tn7 insertion (for Tn7 mutant integration into genome) - 3'	gcttggttgaatgccagttt

Table 2-2: Primers Used In This Chapter (Continued).

Primer	Purpose	Sequence (5' to 3')
ELO106	Use with ELO112 to amplify region surrounding *spt6-481* and *spt6-162* Tn7 insertions (for Tn7 mutant integration into genome) - 5'	ccagacaaaggaggatccaa
ELO108	Use with ELO109 to amplify region surrounding *spt6-76* Tn7 insertion (for Tn7 mutant integration into genome) - 5'	tgaggcaagaaaagtgcaag
ELO109	Use with ELO108 to amplify region surrounding *spt6-76* Tn7 insertion (for Tn7 mutant integration into genome) - 3'	tggaatttttggatccctga
ELO112	Use with ELO106 to amplify region surrounding *spt6-481* and *spt6-162* Tn7 (insertions (for Tn7 mutant integration into genome) - 3'	ggtttcaagagcccaactca
ELO115	Use with ELO116 to amplify region surrounding *spt6-487* Tn7 insertion (for Tn7 mutant integration into genome) - 5'	gcggtgatttgcaagtcttt
ELO116	Use with ELO115 to amplify region surrounding *spt6-487* Tn7 insertion (for Tn7 mutant integration into genome) - 3'	ttcaagtcccaaatggaagg
ELO118	Use with ELO119 to amplify region surrounding *spt6-19, 13*, and *160* Tn7 insertions (for Tn7 mutant integration into genome) - 5'	ttccgtaaactggcattcaa

Table 2-2: Primers Used In This Chapter (Continued).

Primer	Purpose	Sequence (5' to 3')
ELO119	Use with ELO118 to amplify region surrounding *spt6-19, 13*, and *160* Tn7 insertions (for Tn7 mutant integration into genome) - 3'	ccaggaaataaccagtgttcg
FO6851	Use with FO6852 to insert *TAP-NATMx* (or any pFA6a-based tag) into *SPT6* - 5'	aaaatctaacagtagtaagaatagaatgaacaactacc gtcggatccccgggttaattaa
FO6852	Use with FO6851, ELO33, or ELO34 to insert *TAP-NATMx* (or any pFA6a-based tag) into *SPT6* - 3'	ataataaaattaataataacaatggacactacatacg catgaattcgagctcgtttaaac

Table 2-3: Yeast Strains Used In This Chapter.

Strain	Purpose	Genotype
FY653	Control	*MATα his4-912δ lys2-128δ ura3-52 leu2Δ1*
FY857	Control strain and Spn1 project strain construction	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with plasmid pCC11 (*URA3 SPT6*$^{+}$)
FY2180	Control for spot tests and Western blot analysis	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 FLAG-spt6-1004*
FY2796	SH2 project Western blot and tetrad dissection analysis	*MAT***a***/MATα his3Δ200/" leu2Δ1/" lys2-128δ/" trp1Δ63/" ura3-52/" KanMx-GAL1pr-FLO8-HIS3/" SPT6::TAP-NatMx/SPT6* (Diploid strain with one copy of wild-type *SPT6* and one copy of TAP-tagged *SPT6* allele.)
FY2797	SH2 project Western blot and tetrad dissection analysis	*MAT***a***/MATα his3Δ200/" leu2Δ1/" lys2-128δ/" trp1Δ63/" ura3-52/" KanMx-GAL1pr-FLO8-HIS3/" spt6ΔTandem::TAP-NatMx/SPT6* (Last 201 codons of *SPT6* deleted and replaced with TAP tag. Diploid strain with one copy of wild-type *SPT6* and one copy of truncated *spt6* allele.)
FY2798	SH2 project Western blot and tetrad dissection analysis	*MAT***a***/MATα his3Δ200/" leu2Δ1/" lys2-128δ/" trp1Δ63/" ura3-52/" KanMx-GAL1pr-FLO8-HIS3/" spt6ΔSH2*$_2$*::TAP-NatMx/SPT6* (Last 101 codons of *SPT6* deleted and replaced with TAP tag. Diploid strain with one copy of wild-type *SPT6* and one copy of truncated *spt6* allele.)

Table 2-3: Yeast Strains Used In This Chapter (Continued).

Strain	Purpose	Genotype
FY2808	SH2 project strain construction	*MAT***a***/MATα his3Δ200/" leu2Δ1/" lys2-128δ/" trp1Δ63/" ura3-52/" KanMx-GAL1pr-FLO8-HIS3/"*
FY2809	SH2 project spot tests	*MATα his3Δ200 leu2Δ1 lys2-128δ trp1Δ63 ura3-52 KanMx-GAL1pr-FLO8-HIS3*
FY2810	SH2 project spot tests	*MATα his3Δ200 leu2Δ1 lys2-128δ trp1Δ63 ura3-52 KanMx-GAL1pr-FLO8-HIS3 SPT6::TAP-NatMx*
FY2811	SH2 project spot tests	*MATα his3Δ200 leu2Δ1 lys2-128δ trp1Δ63 ura3-52 KanMx-GAL1pr-FLO8-HIS3 spt6ΔTandem::TAP-NatMx* (Last 201 codons of *SPT6* deleted and replaced with TAP tag)
FY2812	SH2 project spot tests	*MATα his3Δ200 leu2Δ1 lys2-128δ trp1Δ63 ura3-52 KanMx-GAL1pr-FLO8-HIS3 spt6ΔSH2$_2$::TAP-NatMx* (Last 101 codons of *SPT6* deleted and replaced with TAP tag)
Tn7 dominant strains	Insertion mutant dominant phenotypic analysis	yEL22 with an additional *spt6::Tn7* mutant plasmid
Tn7 recessive strains	Insertion mutant recessive phenotypic analysis	yEL22 without pEL183; has *spt6::Tn7* mutant plasmid instead
yEL22	Insertion mutagenesis strain construction	*MATα KanMx-GAL1pr-FLO8-HIS3 his3Δ200 leu2Δ1 lys2-128δ trp1Δ63 ura3-52 spt6Δ::LEU2* with plasmid pEL183 (*TRP1 SPT6$^+$*)

Table 2-3: Yeast Strains Used In This Chapter (Continued).

Strain	Purpose	Genotype
yEL89 (FY2801)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL183 (*CEN TRP1 FLAG-SPT6*)
yEL90 (Fy2802)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pCK134 (*CEN TRP1 FLAG-spt6-50*)
yEL91 (FY2803)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL190 (*CEN TRP1 FLAG-spt6-YW*)
yEL92 (FY2804)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL203 (*CEN TRP1 FLAG-spt6-Y*)
yEL93 (FY2805)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL207 (*CEN TRP1 FLAG-spt6-W*)
yEL95 (FY2806)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL193 (*CEN TRP1 FLAG-spt6-IF*)
yEL96 (FY2807)	Spn1 project spot tests and co-IP	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 spt6Δ::LEU2* with pEL187 (*CEN TRP1 FLAG-spt6-GG*)
yEL163	Control	*MAT***a** *lys2-128δ his4-912δ ura3-52 SPT6-Nterm-FLAG ygr117c::NatMX*

Table 2-3: Yeast Strains Used In This Chapter (Continued).

Strain	Purpose	Genotype
yEL165	Insertion mutant strain construction	*MAT***a***/MATα lys2-128δ/" his4-912δ/HIS4 his3Δ200/HIS3 ura3-52/ura3Δ0 leu2Δ1/LEU2 KanMx4::GAL1pr-FLO8/FLO8 SPT6-Nterm-FLAG/SPT6 ygr117c::NatMX/YGR117c*
yEL404	Tn7 integrated mutant for spot tests	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-543 ygr117c::NatMX*
yEL406	Tn7 integrated mutant for spot tests and Western blot analysis	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-76 ygr117c::NatMX*
yEL409	Tn7 integrated mutant for spot tests	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-481 ygr117c::NatMX*
yEL412	Tn7 integrated mutant for spot tests and Western blot analysis	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-487 ygr117c::NatMX*
yEL418	Tn7 integrated mutant for spot tests	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-13 ygr117c::NatMX*

Table 2-3: Yeast Strains Used In This Chapter (Continued).

Strain	Purpose	Genotype
yEL421	Tn7 integrated mutant for spot tests	*MATα lys2-128δ his4-912δ or HIS4 his3Δ200 or HIS3 ura3-52 or ura3Δ0 leu2Δ1 KanMx4::GAL1pr-FLO8 Nterm-FLAG-spt6-19 ygr117c::NatMX*

Figure 2-2: Phenotypic analysis of *spt6::Tn7* mutants. A. Recessive phenotypic analysis. In this system, the genomic copy of *SPT6* is deleted (*spt6Δ*; the blue rectangle represents the genomic copy) and covered by a *CEN* plasmid (shown in red) containing the desired *spt6::Tn7* mutation under the control of a galactose-inducible promoter. Spot tests were performed on plates containing galactose (inducing conditions). **B.** Dominant phenotypic analysis. These strains are similar to Part A except that wild-type *SPT6* is present on a second *CEN* plasmid (shown in green). **C.** Integrated phenotypic analysis. The *spt6::Tn7* mutation is integrated into the genome, replacing the wild-type copy of *SPT6*. All strains used for Parts A, B, and C are haploid.

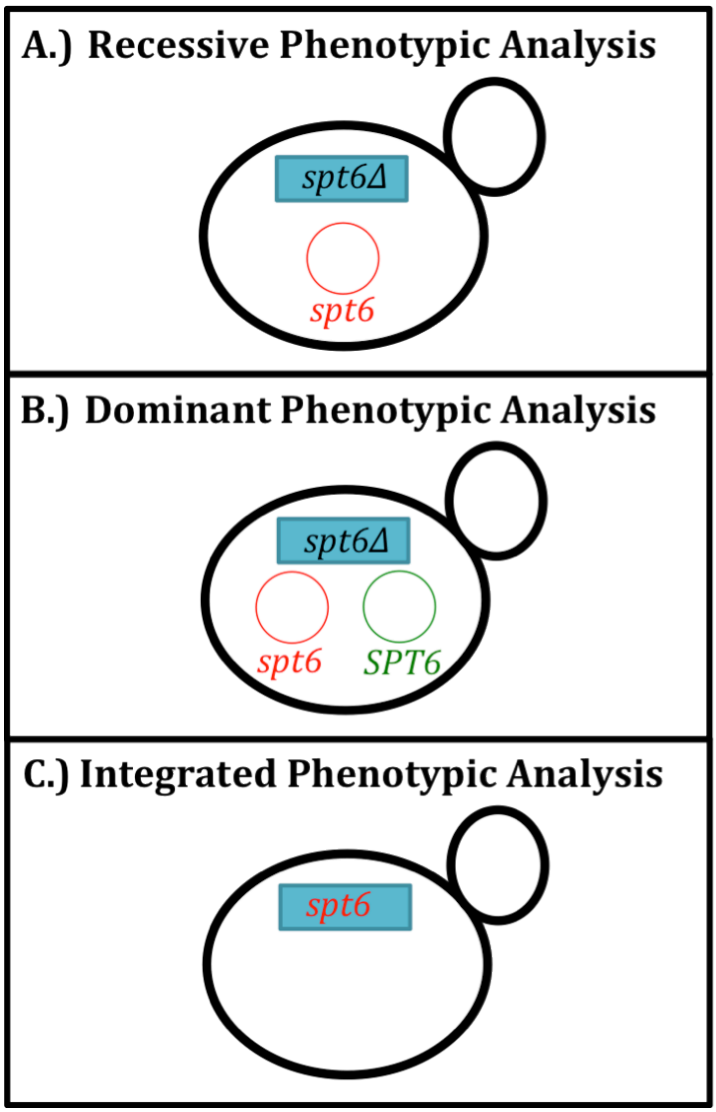

Figure 2-2: Phenotypic analysis of *spt6::Tn7* mutants (Continued).

replacing the gene immediately downstream (*YGR117c*), which has no detectable phenotype (Giaever et al., 2002). The *ygr117cΔ::NatMx* allele was used as a convenient marker for *spt6* mutations. First, a *URA3* open reading frame (ORF) PCR product was inserted at the site of the desired *spt6::tn7* mutant insertion within *SPT6*. Next, PCR products containing the desired *spt6::Tn7* mutation were used to transform the Ura^+ strain, selecting for 5-FOA^R colonies which indicate loss of the *URA3* cassette and integration of the *spt6::tn7* mutation (Boeke et al., 1984). This diploid strain was then sporulated to obtain the desired haploid *spt6::Tn7* strain (Figure 2-2C) (Rose et al., 1990). Mutations were confirmed by sequencing.

To construct *spt6* mutants lacking either one or both SH2 domains, two *spt6* mutants were constructed which encoded for Spt6 C-terminal truncations. All mutant and wild-type constructs also encoded a TAP tag at the C-terminus of *SPT6* to allow for Western blot analysis of Spt6 protein levels. The *spt6-Tandem::TAP* allele is deleted for the last 201 codons of *SPT6*, encoding the two SH2 domains. The *spt6-ΔSH2$_2$::TAP* allele is deleted for the last 101 codons of *SPT6*, encoding the more C-terminal $SH2_2$ domain. All TAP-tagged alleles were created by transforming a *TAP-NatMx* cassette (amplified from pFA6a-C-TAP4-natMX6; Table 2-1) (Van Driessche et al., 2005)) into the diploid strain FY2808. Nat-resistant transformants were isolated, and the TAP-tagged constructs were confirmed by PCR and sequencing.

Sporulation and tetrad dissection to analyze haploid strains that contained the SH2 deletions were performed by standard procedures (Rose et al., 1990). For *SPT6::TAP* (full-length wild-type *SPT6* fused to TAP), cells behaved identically to wild-type cells (They grew at the wild-type growth rate, and no *spt6* mutant phenotypes were detected).

Spot Tests

For all spot tests, yeast strains were grown to saturation in YPD, serially diluted 10-fold, and spotted onto the indicated media (Cheung et al., 2008). The one exception to this was the *spt6::Tn7* dominant phenotype analysis where strains were grown in SC-Ura-Trp to prevent plasmid loss. To analyze the *spt6::Tn7* insertion mutants, the strains were derived from yEL22 and yEL165. Spot tests were performed on the media conditions listed in Table 2-4 (Hampsey, 1997). The Spt$^-$ (**S**u**P**pression of **T**y insertion) phenotype refers to suppression of the Lys$^-$ phenotype of the mutation, *lys2-128δ*, caused by a Ty LTR insertion in the *LYS2* gene (Fassler and Winston, 1988; Simchen et al., 1984). All *spt6::Tn7* spot tests were performed on media containing galactose which causes over-expression of the *spt6::Tn7* mutants. YPD, YPGal, SC-His, SC-Lys, SC-Ura, and SC-Trp were made as described previously (Rose et al., 1990).

To analyze the *spt6* SH2 deletion mutants, the strains used were FY2809, FY2810, FY2811, and FY2812. For Spn1-binding mutant analysis, the strains used contained the *spt6Δ::LEU2* allele in the genome covered by a *CEN* plasmid containing either FLAG-tagged wild-type *SPT6* (yEL89), *spt6-50* (yEL90), *spt6-YW* (yEL91), *spt6-Y* (yEL92), *spt6-W* (yEL93), *spt6-IF* (yEL95), or *spt6-GG* (yEL96). Spot tests were performed on the following media: YPD, YPD at 37 °C, YPD at 16 °C, SC-His, SC-Lys, YPD with 150mM hydroxyurea, YPD with 13μg/ml phleomycin, YPD with 15mM caffeine, and 0.01% methyl methanesulfonate (MMS).

Table 2-4: Summary of mutant phenotypes tested. The Spt$^-$ (**S**u**P**pression of **T**y insertion) phenotype refers to suppression of the Lys$^-$ phenotype caused by the Ty LTR insertion mutation, *lys2-128δ*. All *spt6::Tn7* spot tests were performed on media containing galactose which causes over-expression of the *spt6::Tn7* mutants. All plates were incubated on YPGal at 30°C unless otherwise indicated. (Ausubel et al, 1987; Hampsey, 1997; Rose et al, 1990)

Phenotype	Media Conditions	Predicted defect
Spt$^-$	SC-Lys galactose	Transcription regulation defect
Heat sensitivity	YPGal, grown at 37°C	General protein defect in Spt6
Cold sensitivity	YPGal, grown at 16°C	Complex assembly defect
Cryptic initiation (*FLO8-HIS3* reporter)	SC-His galactose	Open chromatin structure
Cryptic initiation at 37°C (*FLO8-HIS3* reporter)	SC-His galactose, grown at 37°C	Open chromatin structure (in a sensitized background)
Hydroxyurea sensitivity	YPGal + 150mM hydroxyurea	DNA replication defect
Mycophenolic acid (MPA) sensitivity	SC-Ura galactose + 25µg/ml MPA	Transcription elongation defect
UV irradiation sensitivity	YPGal + 50ergs/mm^2 UV irradiation	DNA damage repair defect (base excision repair)
Methyl methanesulfonate (MMS) sensitivity	YPGal + 0.05% (v/v) MMS	DNA damage repair defect (base excision repair)
Phleomycin sensitivity	YPGal + 7µg/ml phleomycin	DNA damage repair defect (double-strand break repair)
Rapamycin sensitivity	YPGal + 0.1µg/ml rapamycin	Tor kinase signaling pathway defect
Nocodazole sensitivity	YPGal + 7µg/ml nocodazole	Microtubule function defect
Thiabendazole sensitivity	YPGal + 7µg/ml thiabendazole	Microtubule function defect

Western Blot Analysis

Analysis of FLAG-tagged or TAP-tagged proteins by Western analysis was done as previously described (Cheung et al., 2008). This was done for strains FY653, yEL163, FY2180, yEL406, yEL412, FY2796, FY2797, and FY2798. The FLAG tag and TAP tag were detected using the M2 α-FLAG antibody (1:1000 dilution; Sigma F3165) and the peroxidase α-peroxidase antibody (1:5000 dilution; Sigma P1291), respectively. Spt6 antiserum was a generous gift from Laura McCollough and Tim Formosa (1:2000 dilution in TBST). Pgk1 was used as a loading control and visualized with α-Pgk1 antiserum (Invitrogen 459250).

Co-immunoprecipitations

Co-immunoprecipitations (co-IPs) were performed as previously described (Zhang et al., 2008). Briefly, cell lysates were prepared from haploid strains containing either untagged wild-type Spt6 (FY857), FLAG-tagged Spt6 (yEL89), Spt6-YW (yEL91), Spt6-Y (yEL92), Spt6-W (yEL93), Spt6-IF (yEL95), or Spt6-GG (yEL96) (Cheung et al., 2008). After pull-down with α-FLAG antibody coupled to Protein G beads, samples were probed for the presence of Spt6 (M2 α-FLAG antibody (1:1000 dilution; Sigma F3165)) and Spn1 (α-Spn1 antibody, courtesy of Catherine Radebaugh and Laurie Stargell) (Zhang et al., 2008).

GST Pull-down Experiments, Phosphospecificity Analysis, and Protein Structure Determination

These experiments were performed by our collaborators. Detailed methods can be found in our collaborative publications (Diebold et al., 2010a; Diebold et al., 2010b). It should be noted that the crystal structure of the tandem SH2 domains was solved in the

unicellular eukaryotic parasite *Antonospora locustae*. This organism has shorter versions of many eukaryotic proteins while still retaining the essential organization of orthologs seen in other organisms, such as *S. cerevisiae* (Diebold et al., 2010a; Diebold et al., 2010b; Keeling, 2001). Use of *A. locustae* made the SH2 domain structure determination possible whereas all previous attempts to crystallize Spt6 in *S. cerevisiae* had failed. For the Spn1-Spt6 NTD structure, *Encephalitozoon cuniculi* was used for similar reasons.

Results

Isolation and Analysis of Plasmid-borne Insertion Mutations in *SPT6*

To understand the roles of the different domains of Spt6, I have performed a large-scale random mutagenesis of *SPT6* using *in vitro* transposon-based insertion mutagenesis (Biery et al., 2000b). This method introduces random fifteen base pair insertions into a *CEN* plasmid with a DNA construct encoding MYC-tagged Spt6 driven by the *GAL1* galactose-inducible promoter (Figure 2-1; M. Gupta, unpublished data). The *SPT6* gene was put under control of a galactose-inducible promoter in order to be able to control Spt6 protein levels and to identify *spt6* mutations that enhanced or impaired the over-expression phenotype of Spt6 (Clark-Adams and Winston, 1987). Of the six possible reading frames of the insertions, two will result in a termination codon while four will insert a sequence encoding five amino acids into the *SPT6* open reading frame. I successfully isolated sixty-seven mutations positioned randomly throughout *SPT6* with at least one insertion in every region of the gene encoding a putative domain (Figure 2-3). All of the mutations were analyzed by restriction digestion and DNA sequencing (Materials

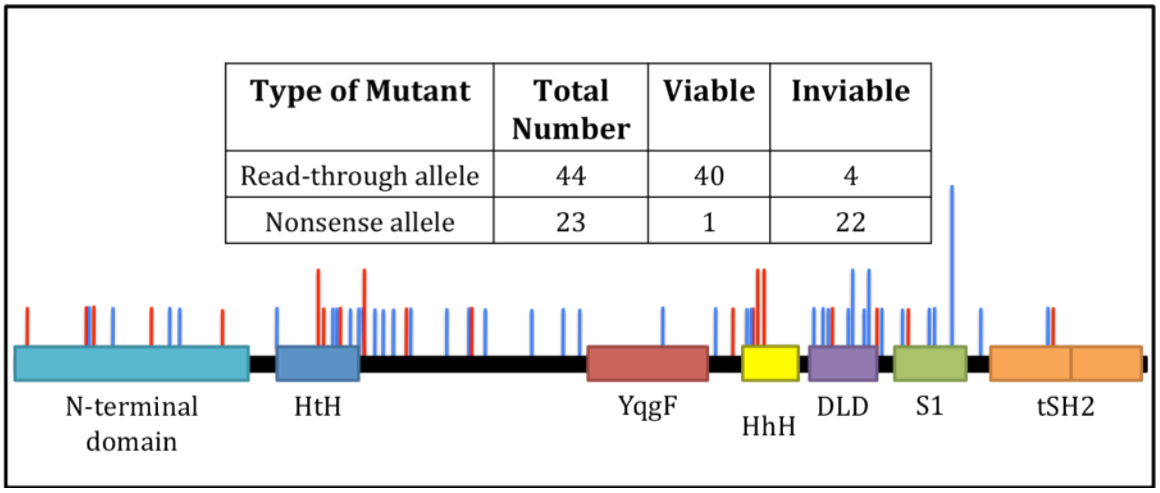

Figure 2-3: Overview of insertions within Spt6. The locations of the identified read-through alleles (blue) and truncation alleles (red) are shown above the diagram of Spt6 domains. The height of the bars indicates the number of insertions at that location (either one, two, or four). In six cases, two insertions are found at the same location and, in one case, four insertions are found at the same location. As expected, approximately one third of insertion mutations introduce a stop codon. The vast majority of read-through mutations allow viability, while all but one of the nonsense alleles cause inviability.

and Methods). Of the sixty-seven mutations, forty-four are read-through alleles and twenty-three introduce a translation termination codon.

To determine the possible recessive mutant phenotypes caused by each *spt6::Tn7* allele, each mutant plasmid was screened by plasmid shuffle (Figure 2-3) as described in Materials and Methods. Of the sixty-seven insertion mutants, forty-one are viable (forty encode read-through alleles and one encodes a C-terminal truncation) and twenty-six are inviable (twenty-two encode truncations and four encode read-through alleles). Viable mutants were analyzed by serial dilution spot tests for a set of thirteen phenotypes (Table 2-4).

The results of the phenotypic screens are summarized in Table 2-5. Several mutants are pleiotropic with a number of mutant phenotypes, such as mutants *spt6-101, 313,* and *201* (Table 2-5A). However, there are also several mutants that have one primary phenotype. For example, mutants *spt6-76, 481, 162*, and *13* are primarily sensitive to only high temperature, MMS, mycophenolic acid, or phleomycin, respectively (Table 2-5A). This is particularly intriguing because the vast majority of preiously-known *spt6* mutant alleles are highly pleiotropic, making it difficult to determine any specific effects of the mutation (Kaplan, 2002).

Interestingly, there are two examples of pairs of insertion mutants at adjacent amino acid positions that have very different phenotypes (for example, one behaving like wild type and the other having strong mutant phenotypes). Examples of this are found in the region encoding the DLD domain and include mutants *spt6-313* and *spt6-347* (at

Table 2-5: Recessive and dominant phenotype summary of *spt6* insertion mutants.

A. Recessive phenotype summary. The scoring of the phenotypes for each viable *spt6* insertion mutation is shown. The mutant name is listed in the far left column and the amino acid location of the insertion in the Spt6 protein is listed in the second column. Each phenotype was given a numerical score of 0 though 5 (0 being no growth and 5 being maximum growth). All significant phenotypes are highlighted in color. The colors correspond to the strength and type of phenotype (see Table 2-5 key on the following page). The media conditions are listed at the top. Phenotype scores are listed for the day shown in the second row. Details regarding media conditions used can be found in Table 2-4. **B.** Dominant phenotype summary for all *spt6* insertion mutations. The phenotypes are scored and organized similarly to Table 2-5A. In the far left column, read-through mutations are shown in purple and nonsense alleles are shown in orange. All media plates used for Table 2-5B were SC-Ura-Trp galactose unless otherwise indicated. All other conditions were similar to Table 2-5A. In regards to the Spt^- (SC-Lys) phenotype in the dominant phenotype analysis, the Spt^- phenotype is the opposite of what is seen in a recessive phenotype analysis. Wild-type *SPT6* causes an Spt^- phenotype when over-expressed in the dominant phenotype analysis context. If an over-expressed *spt6* mutant behaves differently (for example, if it does not have an Spt^- phenotype), this is scored as a mutant phenotype. One phenotype for mutant *spt6-162* is listed as "NA" (not available) due to contamination which obscured that spot test row. (Abbreviations: AA, amino acid position; WT, wild type; MPA, Mycophenolic acid; HU, Hydroxyurea; Phleo, Phleomycin; MMS, Methyl methanesulfonate; Rapa, Rapamycin; Noco, Nocodazole; TBZ, Thiabendazole; NA, not available)

Table 2-5: Recessive and dominant phenotype summary of *spt6* insertion mutants (Continued; remainder of Table 2-5 on following pages).

Key		Weakly resistant (≥ 1 spot difference in growth from pMG2 (WT))
		Strongly resistant (≥ 2 spot difference in growth from pMG2 (WT))
		Weakly sensitive (≥ 1 spot difference in growth from pMG2 (WT))
		Strongly sensitive (≥ 2 spot difference in growth from pMG2 (WT))
		Read-through allele
		Nonsense allele

Table 2-5: Recessive and dominant phenotype summary of *spt6* insertion mutants (Continued).

A.)												SC-His		
		MPA	UV	HU	Phleo	MMS	Rapa	Noco	TBZ	SC-Lys	SC-His	37°C	37°C	16°C
Mutant	AA	Day 4	Day 4	Day 4	Day 4	Day 4	Day 4	Day 7	Day 7	Day 4	Day 4	Day 4	Day 4	Day 7
pMG2	WT	5	5	4.5	5	0	4.5	0	3	4	1	0	5	4.5
543	126	5	0	3	3	0	4.5	0	1	4	0	0	3	4.5
101	201	5	5	2	3	0	4.5	0	4	4	1	0	3	4.5
411	211	5	5	4.5	5	0	4.5	0	4.5	4	0	0	5	4.5
585	337	5	5	4.5	5	0	4.5	0	4.5	4	0	0	5	4.5
76	409	5	5	5	5	0	4	1.5	4	4	0	0	0	4.5
390	411	5	5	5	5	0	4	1.5	4	4	0	0	0	4.5
492	430	5	5	4	5	0	4.5	0	4	4	0	0	5	4.5
488	446	5	5	5	5	0	4.5	0	4	4	0	0	5	4.5
423	447	5	5	5	5	0	4.5	1	4	4	0	0	5	4.5
181	463	5	5	4	5	0	4.5	0	4	4	0	0	5	4.5
402	475	5	5	5	5	0	4.5	1	4.5	4	0	0	5	4.5
425	489	5	5	4	5	0	4.5	0	4	4	0	0	5	4.5
481	556	5	5	3.5	4	1	4	0	3	4	0	0	4.5	4
162	588	4	5	3.5	4.5	2	2	0	5	3	0	0	5	4
173	606	5	5	5	5	1.5	4	0	4	3	0	1	4	4
114	664	5	5	4	5	2	4	0	4	4	0	0	5	4
148	834	5	5	3.5	4.5	2	4	0	3	4	0	0	5	4
103	900	5	5	3.5	4.5	2	4	0	3	4	0	0	5	4
182	944	5	5	3.5	4.5	2	4	0	3	4	0	0	5	4
19	945	5	5	4	5	2	4	0	1	4.5	0	1.5	5	4
151	1028	5	5	4	4.5	2	4	0	3	4	0	0	5	4
313	1041	5	5	3	3	1	4	0	1.5	3	0	0	4	4
347	1046	5	5	3.5	4.5	2	4	0	2	4	0	0	5	4
487	1047	5	5	5	5	3.5	4	0	3	5	0	0	5	4.5
591	1079	5	5	5	5	2	4	0	1.5	4	0	0	5	5
3	1083	5	5	4	4.5	1	4	0	2	4	0	0	5	5
22	1083	5	5	4	4.5	1	4	0	2	4	0	0	5	5
497	1095	5	5	4	4.5	1	4	0	1.5	4	0	0	5	5
13	1098	5	5	3	2.5	0	4	0	0	3	0	0	4.5	5
160	1099	5	5	4.5	5	1.5	4	0	3	4.5	0	0	5	5
323	1114	5	5	5	5	1.5	4	1	3	4.5	0	0	5	5
463	1143	5	5	4.5	5	1	4	0	2	4	0	0	5	5
465	1179	5	5	4.5	5	1	4	0	3	4	0	0	5	5
332	1183	5	5	5	5	2	4	1	3	4	0	0	5	5
135	1206	5	5	4.5	5	1.5	4	0	2.5	4	0	0	5	5
138	1206	5	5	4.5	5	1.5	4	0	3	4	0	0	5	5
569	1206	5	5	4	5	1.5	4	0	3	4	0	0	5	4.5
547	1207	5	5	4	5	1.5	4	0	3	4	0	0	5	4.5
454	1247	5	5	4	4.5	1.5	4	0	1	4	0	0	4	4.5
429	1330	5	5	4	5	1	3	0	3	4	0	0	5	4.5
201	1341	5	5	0	1	0	1.5	1	0	5	5	0	0	1.5

Table 2-5: Recessive and dominant phenotype summary of *spt6* insertion mutants (Continued).

B.)		MPA	UV	HU	Phleo	MMS	SC-Lys	SC-His	37°C	16°C
Mutant	AA	Day 4	Day 4	Day 4	Day 4	Day 4	Day 3	Day 4	Day 4	Day 4
pMG2	WT	5	5	1	2	2	3	0	5	3
389	16	5	5	3	2	2	1	0	5	3
602	91	5	5	3	2	2	1	0	5	3
601	92	5	5	2	2	2	3	0	4.5	3
513	94	5	5	3	2	2	1	0	5	3
543	126	5	1	2	2	0	3	0	4.5	3
478	178	5	5	3	2	2	1	2.5	5	3
101	201	5	5	1	2	1	3	0	4.5	3
411	211	5	5	2	2	2	3	0	5	3
564	266	5	5	2	2	2	3	0	4.5	3
319	267	5	5	2	2	2	3	0	5	3
585	337	5	5	1.5	2	2	3	0	5	3
102	390	5	5	3	2	2	1	0	5	3
438	390	5	5	3	2	2	1	0	5	3
549	399	5	5	3	2	2	1	0	5	3
76	409	4.5	5	3	1.5	2	1.5	0	5	3
390	411	4.5	5	3	1.5	2	1	0	5	3
164	421	4.5	5	3	1.5	2	1	0	5	3
492	430	4.5	5	2	1.5	2	3	0	5	3
488	446	4.5	5	2	1.5	2	3.5	0	5	3
423	447	4.5	5	2	1.5	2	3.5	0	5	3
150	448	4.5	5	3	1.5	2	2.5	0	5	3
385	448	4.5	5	3	1.5	2	2.5	0	5	3
181	463	4.5	5	2	1.5	2	3.5	0	5	3
402	475	4.5	5	2	1.5	2	3.5	0	5	3
425	489	4.5	5	2	1.5	1.5	3	0	5	3
73	506	4.5	5	3	1.5	2	2.5	0	5	3
285	510	4.5	5	2	1.5	2	3	0	5	3
481	556	4.5	5	2	1.5	2	3	0	5	3
240	588	4.5	4.5	3.5	1	1.5	0	0	5	3
162	588	4.5	4.5	2	1	2	NA	0	5	3
173	606	4.5	4.5	3	1	1.5	1.5	0	5	3
114	664	4.5	4.5	2	1	1.5	1.5	0	5	3
184	706	4.5	4.5	3	1	1.5	1.5	0	5	3
507	725	4.5	4.5	3	1	2	1.5	0	5	3
148	834	4.5	4.5	2	1	1.5	2	0	5	3
487	900	4.5	4.5	2	1	1.5	2	0	5	3
388	926	4.5	4.5	4	1	2	1.5	0	5	3
103	944	4.5	4.5	2	1	1.5	2	0	5	3
182	945	4.5	4.5	2	1	1	2	0	5	3
563	965	4.5	4.5	3	1	1.5	1	0	5	3
582	965	4.5	4.5	3	1	1.5	1	0	5	3
455	967	4.5	4.5	3	1	1.5	1	0	5	3

Table 2-5: Recessive and dominant phenotype summary of *spt6* insertion mutants (Continued).

B.) (Continued)		MPA	UV	HU	Phleo	MMS	SC-Lys	SC-His	37°C	16°C
Mutant	AA	Day 4	Day 4	Day 4	Day 4	Day 4	Day 3	Day 4	Day 4	Day 4
19	1028	5	4	2	1	1	3	0	5	2
151	1041	5	4.5	2	1	1	3	0	5	2
313	1046	5	4.5	2	1	1	3	0	5	2
347	1047	5	4.5	2	1	1	3	0	5	2
591	1079	5	4.5	2	1	1	3	0	5	2
3	1083	5	4.5	2	1	1	3	0	5	2
22	1083	5	4.5	2	1	1	3	0	5	2
497	1095	5	4.5	2	1	1	3	0	5	2
13	1098	5	4.5	2	1	1	3	0	4.5	2
160	1099	5	4.5	2	1	1	3	0	5	2
379	1112	5	4.5	2	1	1	3	0	5	2
323	1114	5	4.5	2	1	1	3	0	5	2
463	1143	5	4	2	1	1	3	0	5	2
417	1151	5	4.5	3	1	1	2	0	5	2
465	1179	4.5	5	2	0	1	3	0	5	2
332	1183	4.5	5	2	0	1	3	0	5	2
135	1206	4.5	5	2	0	1	3	0	5	2
138	1206	4.5	5	2	0	1	2.5	0	5	2
569	1206	4.5	5	2	0	1	2.5	0	5	2
547	1207	4.5	5	2	0	1	2.5	0	5	2
454	1247	4.5	5	2	0	1	3	0	5	2
429	1330	4.5	5	2	0	1	3	0	5	2
201	1341	4.5	5	3	0	1	3	0	5	2

codons encoding residues 1046 and 1047, respectively), as well as *spt6-13* and *spt6-160* (at nearby codons encoding residues 1098 and 1099, respectively). Analysis of the amino acid sequences inserted in these mutants indicates that *spt6-313* and *spt6-347* contain sequences encoding the identical five amino acids (methionine, phenylalanine, lysine, histidine, glutamic acid), while the *spt6-13* and *spt6-160* insertions are in different reading frames and therefore encode different amino acid sequences (valine, phenylalanine, lysine, histidine, leucine, or glutamic acid, cysteine, leucine, aparagine, isoleucine, respectively). The significance of these phenotypic differences in mutants with insertions at adjacent codons is not clear.

In addition, I also screened for dominant phenotypes as described in Materials and Methods (Figure 2-2B; Table 2-5B). Overall, fewer phenotypes were observed in comparison to the recessive phenotypic analysis. A cluster of six mutations within the region encoding the HtH domain and three mutations within the region encoding the HhH domain all cause strong dominant Spt^- phenotypes. However, as discussed below, many of the strong recessive and dominant phenotypes are not observed upon integration of these mutations into the genome at the *SPT6* locus.

Analysis of *spt6* Insertion Mutations Following Integration

For further analyses, seven *spt6* mutations were chosen that either had particularly unique and interesting phenotypes or that cause one or two strong phenotypes without being pleiotropic (Figure 2-4). These mutants were considered more likely to impair specific aspects of Spt6 function rather than being generally defective. Since the version of *SPT6* on the original plasmid was under the control of the *GAL1* promoter, I integrated

Figure 2-4: Comparison of phenotypes of candidate insertion mutants on plasmids versus when integrated. **A.** A diagram of the location of the candidate transposon insertions selected for integration into the genomic copy of the *SPT6* gene. All are read-through alleles and are shown in blue. **B.** Comparison of the phenotypes of the selected candidate *spt6::Tn7* mutants when on a plasmid and when integrated in the yeast genome. The well-characterized *spt6-1004* mutation, which is a deletion of the HhH domain, is listed for comparison purposes. "Inconsistent" indicates that two identical but individually isolated mutants had different phenotypes on the same spot test plate (for example, one was sensitive to phleomycin while the other was not). All mutants were confirmed by sequencing the entire mutant *spt6* gene, so the reasons for the variable results are not understood. For technical reasons, *spt6-162* could not be integrated into the genomic copy of *SPT6* and therefore was not analyzed.

A.)

B.)

Mutant	Domain	Recessive Phenotypes When on a Plasmid	Phenotypes When Integrated into the Genomic *SPT6* locus
spt6-543	NTD	Sensitive to UV, phleomycin, 37°C	Inconsistently sensitive to phleomycin
spt6-76	HtH	Sensitive to 37°C	Slow growth, Spt$^-$, sensitive to UV, HU, phleomycin, MMS, MPA, 37°C
spt6-481		Sensitive to MMS	Inconsistently sensitive to phleomycin
spt6-162		Sensitive to MPA	Not available
spt6-487		Resistant to MMS	Spt$^-$, sensitive to phleomycin, 37°C
spt6-19	DLD	Cryptic initiation at 37°C	Inconsistently sensitive to phleomycin
spt6-13	DLD	Sensitive to phleomycin	Inconsistent cryptic initiation and sensitivity to phleomycin
spt6-1004	HhH	Not available	Spt$^-$, cryptic initiation, sensitive to UV, HU, phleomycin, MPA, 37°C

Figure 2-4: Comparison of phenotypes of candidate insertion mutants on plasmids versus when integrated (Continued).

these mutations into the genome, replacing the wild-type copy of *SPT6*. The phenotypes of these mutant strains were tested to determine whether the integrated mutations cause phenotypes similar to what was observed when they were plasmid-borne.

Surprisingly, the integrated mutants show a very different spectrum of phenotypes (Figure 2-4B). For example, *spt6-481* and *spt6-13*, which on plasmids had been shown to cause sensitivity to MMS and phleomycin, respectively, did not cause any strong phenotype when integrated. Interestingly, two of the integrated mutations cause a number of unexpected phenotypes (Figure 2-4). The *spt6-76* mutation, which causes sensitivity to 37°C when on a plasmid, causes slow growth, an Spt$^-$ phenotype, and sensitivity to UV irradiation, HU, phleomycin, MMS, MPA, and 37°C when integrated. The *spt6-487* mutation, which causes resistance to MMS when on a plasmid, confers sensitivity to 37°C, phleomycin, and has an Spt$^-$ phenotype when integrated. These two mutations are located in regions of *SPT6* with no known function: *spt6-76* is located in the area encoding the HtH domain while *spt6-487* is found in the region encoding the uncharacterized linker between the YqgF homology domain and the HhH domain. Interestingly, both of these mutants differ from previously isolated *spt6* mutants (Kaplan, 2002). For example, neither of these insertion mutations causes cryptic initiation. Overall, it is clear that the selected mutants behave differently when integrated versus when over-expressed on a plasmid.

To determine if Spt6 protein levels are affected in the most pleiotropic of the integrated mutants, Western blot analysis was performed on the *spt6-76* and *spt6-487* mutants. My results (Figure 2-5) show that the amount of Spt6 protein present in both mutants is greatly decreased, as the Spt6-76 protein is undetectable by Western blot, and

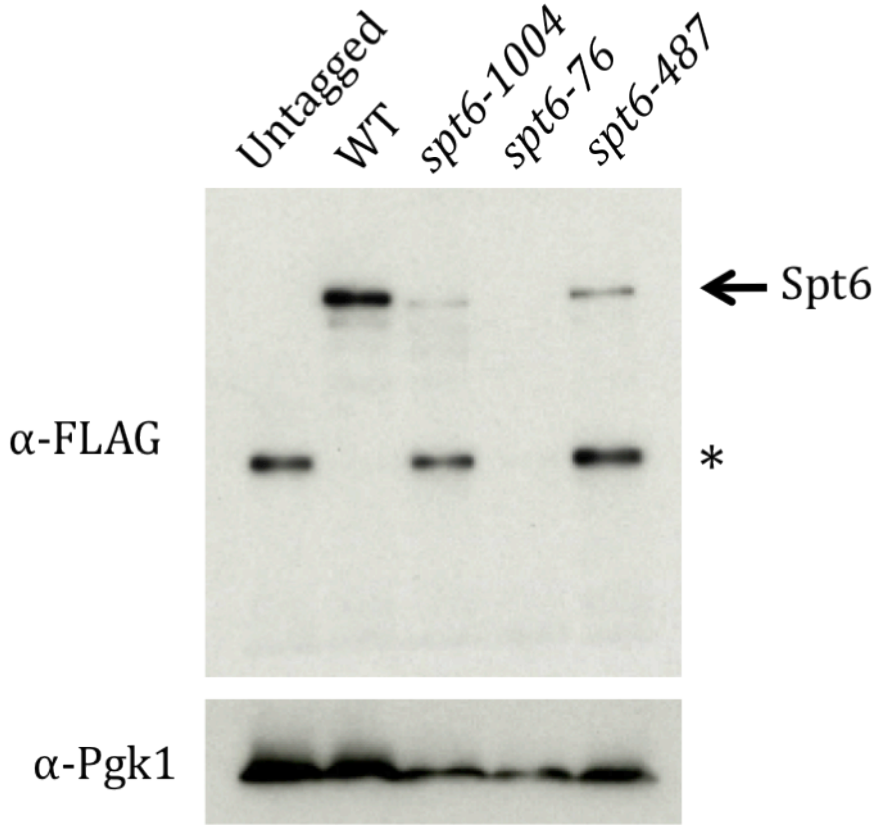

Figure 2-5: Western blot analysis of Spt6 protein levels in *spt6-76* and *spt6-487* mutants. The top panel shows a Western blot probed with α-FLAG antibody to determine the level of FLAG-tagged Spt6 proteins. The top band is FLAG-tagged Spt6. The lower band (*) is a nonspecific band that only appears in some lanes for reasons I do not understand. The bottom panel shows the Pgk1 loading control.

there is a decreased level of Spt6-487, although not as much as the well-studied Spt6-1004 mutant protein (Kaplan et al., 2005). In fact, the number of mutant phenotypes observed for *spt6-76* and *spt6-487* correlates with their Spt6 mutant protein levels. Of the mutants analyzed by Western blot, *spt6-76* has the largest number of phenotypes and the lowest level of Spt6 protein. Overall, this suggests that the *spt6-76* and *spt6-487* pleiotropic loss-of-function mutant phenotypes are due to low Spt6 protein levels.

Lastly, to further address the difference in phenotypes between the integrated and over-expressed plasmid-borne *spt6* mutants, I attempted to determine whether the original phenotypes observed in the *spt6::Tn7* plasmid strains were dependent on the mutant plasmid. Using a 5-FOA plasmid shuffle, the *spt6::Tn7* plasmid was removed from the strains used for the dominant phenotype analysis, leaving only a wild-type *SPT6* plasmid. This was done for the mutants I had integrated into the genomic *SPT6* locus (*spt6-543, 76, 481, 162*, *487, 19,* and *13*), a negative control mutant with no detectable phenotype (*spt6-160*), and two additional mutants with interesting plasmid-based phenotypes (*spt6-101* and *313*). Strains containing either the template wild-type *SPT6* plasmid (pMG2) or a known *spt6* mutant plasmid (pGP26 containing *spt6-140*; Table 2-1) were also tested as negative and positive controls, respectively. I then analyzed the resulting strains by spot tests. If the strains lost their mutant phenotypes after loss of the mutant *spt6::Tn7* plasmid, it would suggest that these phenotypes were in fact dependent on the mutant plasmid. This is what I observed in all cases, with the exception of the strong UV-sensitive phenotype of *spt6-543*. This phenotype remained after loss of the mutant plasmid, suggesting that this phenotype is likely caused by some mutation elsewhere in the genome. However, in general, it appears that the phenotypic discrepancy between the integrated

and plasmid-based mutants cannot be explained by factors independent of the *spt6::Tn7* plasmids. This analysis was not done for the strains used for the recessive phenotype analysis.

In conclusion, the insertion mutations cause a variety of dominant and recessive phenotypes when over-expressed on plasmids. However, these mutations cause little or no phenotype when integrated. In general, no clustering of phenotypes was observed. Two integrated mutations (*spt6-76* and *spt6-487*) cause interesting phenotypes and decreased Spt6 protein levels and are candidates for further study. However, their phenotypes are similar to previously well-characterized *spt6* mutants (for example, *spt6-1004*), and therefore no further work was done on them at this time.

Analysis of SH2 Truncation Mutants

As a more directed approach to perform structure-function analysis of Spt6, I sought to understand the importance of the C-terminal tandem SH2 domains of Spt6. This was done in a collaborative project with Marie-Laure Diebold and Christophe Romier (Diebold et al., 2010b). To study the requirement for the Spt6 SH2 domains, yeast strains containing three alleles (*SPT6::TAP*, *spt6-ΔTandem::TAP*, and *spt6-ΔSH2$_2$::TAP*) were constructed as described in Materials and Methods. These three alleles all add a TAP epitope tag to Spt6 and also result in the deletion of either no Spt6 sequence, both SH2 domains, or the C-terminal SH2 domain, respectively.

To test the *in vivo* requirement for the SH2 domains, thorough phenotypic analysis of these truncation mutants was performed. Haploids containing *spt6-ΔTandem::TAP* or

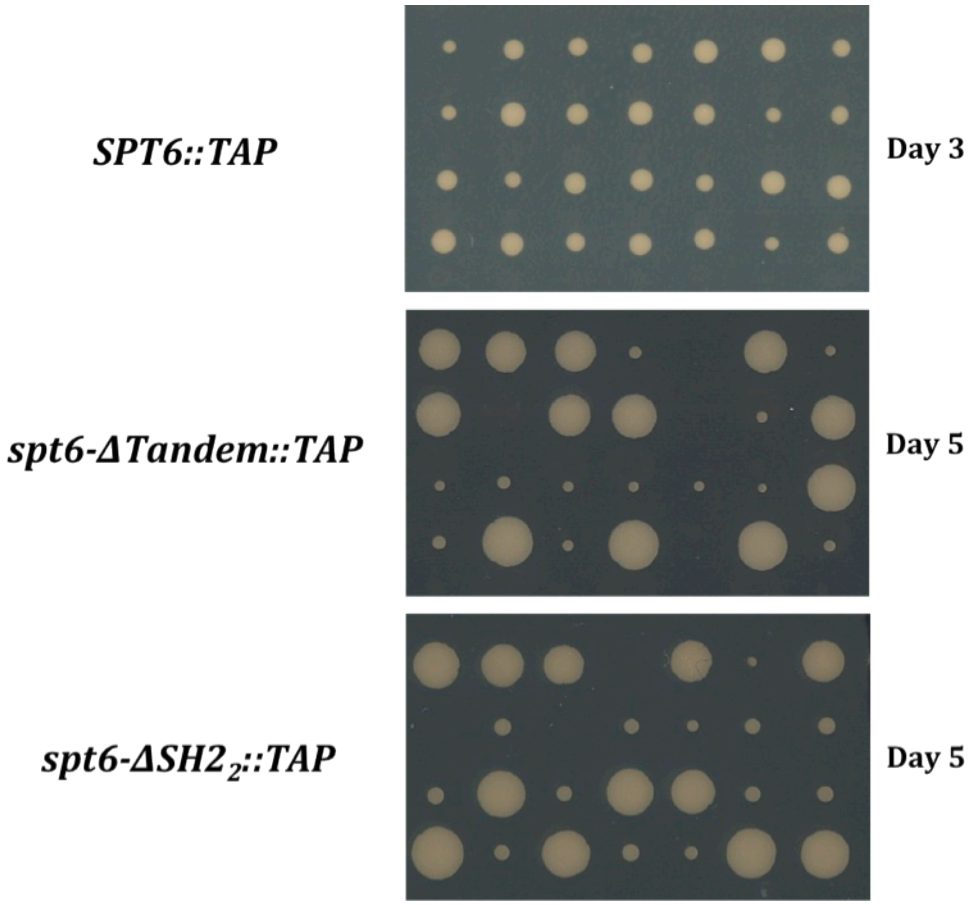

Figure 2-6: Tetrad analysis of SH2 deletion mutants. Tetrad dissection from a diploid yeast heterozygous for either *SPT6::TAP, spt6-ΔTandem::TAP,* or *spt6-ΔSH2$_2$::TAP.* Each column of four colonies represents the four progeny of a single tetrad. Shown are the tetrad dissection plates after incubation at 30°C for either three days (*SPT6::TAP*) or five days (*spt6-ΔTandem::TAP* and *spt6-ΔSH2$_2$::TAP*). For *spt6-ΔTandem::TAP* and *spt6-ΔSH2$_2$::TAP* mutants, all progeny containing the truncations are slow-growing, indicating that *spt6-ΔTandem::TAP* and *spt6-ΔSH2$_2$::TAP* cause a severe growth defect.

spt6-ΔSH2₂::TAP are viable but grow much slower than wild type (Figure 2-6). Thirteen complete tetrads were analyzed for each mutant, with tetrad size and the SH2 deletion co-segregating in all cases except for one wild-type tetrad spore that grew poorly, likely because it became petite.

In addition to poor growth, these truncation mutants also have a number of other phenotypes including Spt$^-$, Ts$^-$, Cs$^-$, cryptic initiation, HU^s, Phleo^s, MMS^s (not shown), and Caf^s (Figure 2-7). Overall, these two mutants are very similar in their phenotypes. The only detectable difference between them is that *spt6-ΔSH2₂::TAP* grows slightly faster than *spt6-ΔTandem::TAP* (Figure 2-6). A possible explanation for this is that the remaining SH2 domain in *spt6-ΔSH2₂::TAP* makes a modest contribution to growth.

To test whether these phenotypes might be caused by reduced Spt6 protein levels, Western blot analysis was performed on diploid strains that are heterozygous for wild-type *SPT6* and one of the truncation mutants. My results show that Spt6 protein is produced at approximately wild-type levels in both truncation mutants (Figure 2-8). Taken together, these results show that loss of either both SH2 domains or only the $SH2_2$ domain severely affects Spt6 function *in vivo* without affecting Spt6 protein levels.

To test for direct interactions between the Spt6 SH2 domains and the RNAPII C-terminal domain (CTD), our collaborators performed GST pull-down studies. Their results demonstrated that both SH2 domains are necessary and sufficient for RNAPII CTD binding *in vitro* (Figure 2-9A) (Diebold et al., 2010b). Mutating the codons encoding predicted phosphopeptide-binding residues within the SH2 domains impairs RNAPII CTD binding but does not eliminate it altogether (Figure 2-9A). This result suggests that either the crucial

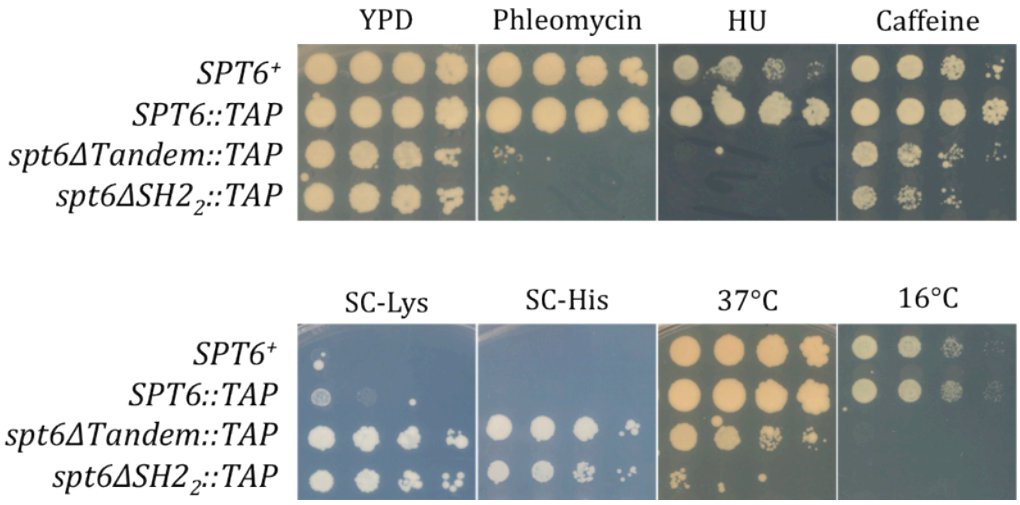

Figure 2-7: Mutant phenotypes of *spt6* SH2 deletion mutants. Each row shows serial dilutions of a yeast strain containing either untagged (*SPT6*$^+$), wild-type TAP-tagged Spt6 (*SPT6::TAP*), or the TAP-tagged truncation mutants (*spt6ΔTandem::TAP* or *spt6ΔSH2*$_2$*::TAP*). The strains were grown to saturation in YPD, serially diluted 10-fold, and spotted on the indicated media. Plates were photographed after 6 days of incubation. All plates were incubated at 30°C unless otherwise indicated. The truncation alleles are HU^s, Phleo^s, Caf^s, and exhibit cryptic initiation at the *FLO8-HIS3* reporter (Cheung et al, 2008). They are also Spt$^-$ (able to grow on medium lacking lysine (SC-Lys) despite the presence of the *lys2-128δ* Ty LTR insertion allele), Ts$^-$ at 37°C, and Cs$^-$ at 16°C. (Abbreviations: HU, hydroxyurea)

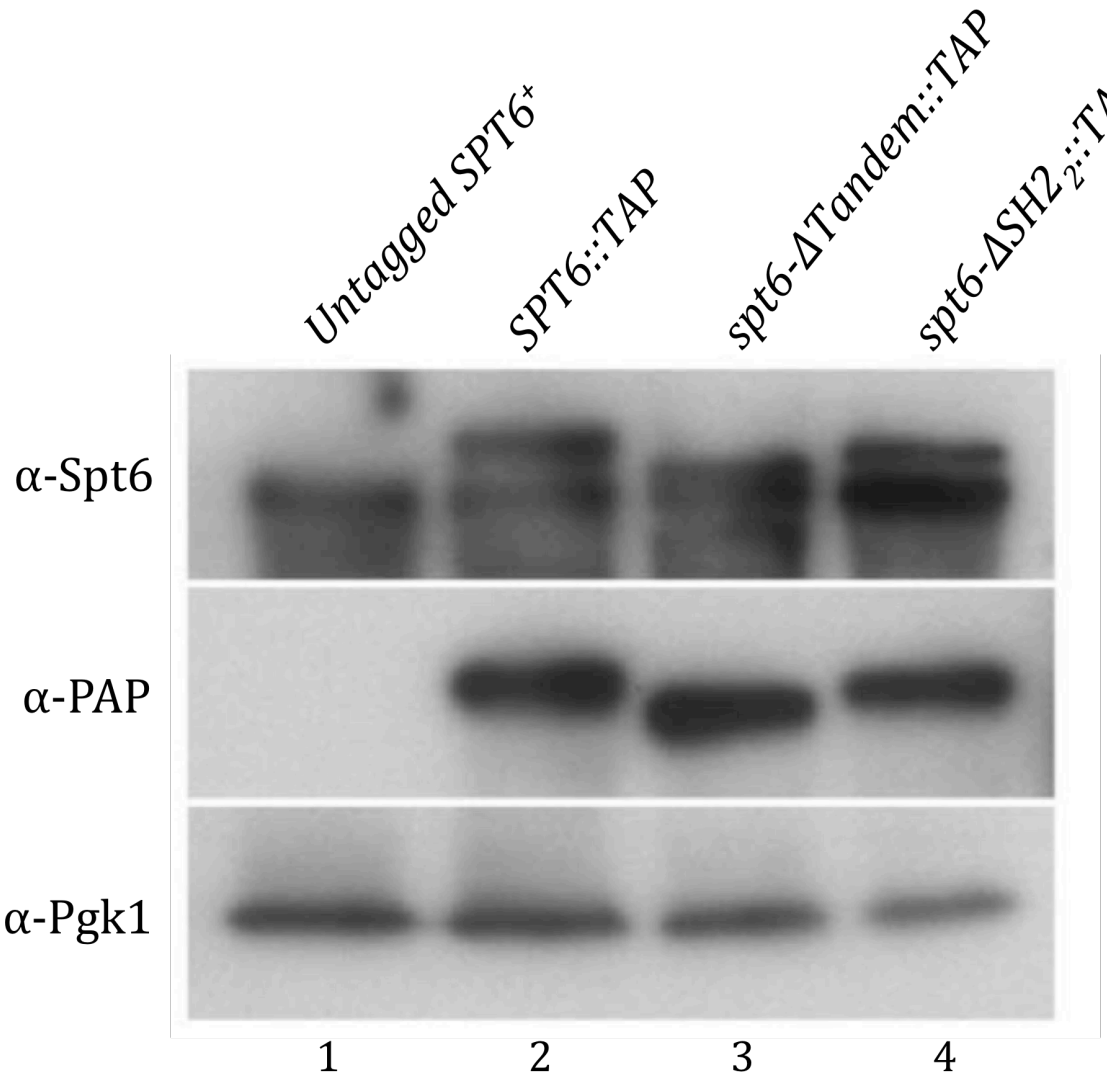

Figure 2-8: Western blot analysis of Spt6 levels in truncation mutants. The top panel shows a blot probed with anti-Spt6 antibody (courtesy of Laura McCollough and Tim Formosa). In this panel, in lanes 2, 3, and 4, two bands are visible because the strains are diploid. The top band is the TAP-tagged Spt6 and the lower band is untagged Spt6. The middle panel is probed with anti-PAP antibody to detect only the TAP-tagged mutant Spt6 proteins, and the bottom panel shows the Pgk1 loading control. The truncation alleles do not cause any significant change in Spt6 protein levels.

Figure 2-9: Role of SH2 domains in binding the RNAPII CTD and analysis of phosphospecificity of the Spt6 tandem SH2 region. A. GST pull-downs were carried out using GST-fused *S. cerevisiae* (*sc*) Spt6 constructs in the presence of yeast extract. Binding of the RNAPII CTD was monitored by α-CTD antibody. The yeast extract control loaded (lane 1) corresponds to 0.1 µl of extract, whereas the quantity loaded after pull-down and washes corresponds to the equivalent of 2.5 µl of extract. Coomassie-stained samples indicate the amount of each GST-tagged Spt6 construct in the input. GST pull-downs were performed on the yeast Spt6 wild-type tandem SH2 domain (lane 2), $SH2_1$ domain (lane 3), $SH2_2$ domain (lane 4), and mutants of phosphate-binding residues from both domains in the context of the entire tandem SH2 domain (lanes 5–7). **B.** Analysis of phosphospecificity using α-RNAPII CTD Ser2P and α-Ser5P antibodies. A slight increase in binding to the serine 2-phosphorylated RNAPII CTD was observed. (This figure is adapted from Diebold et al., 2010b)

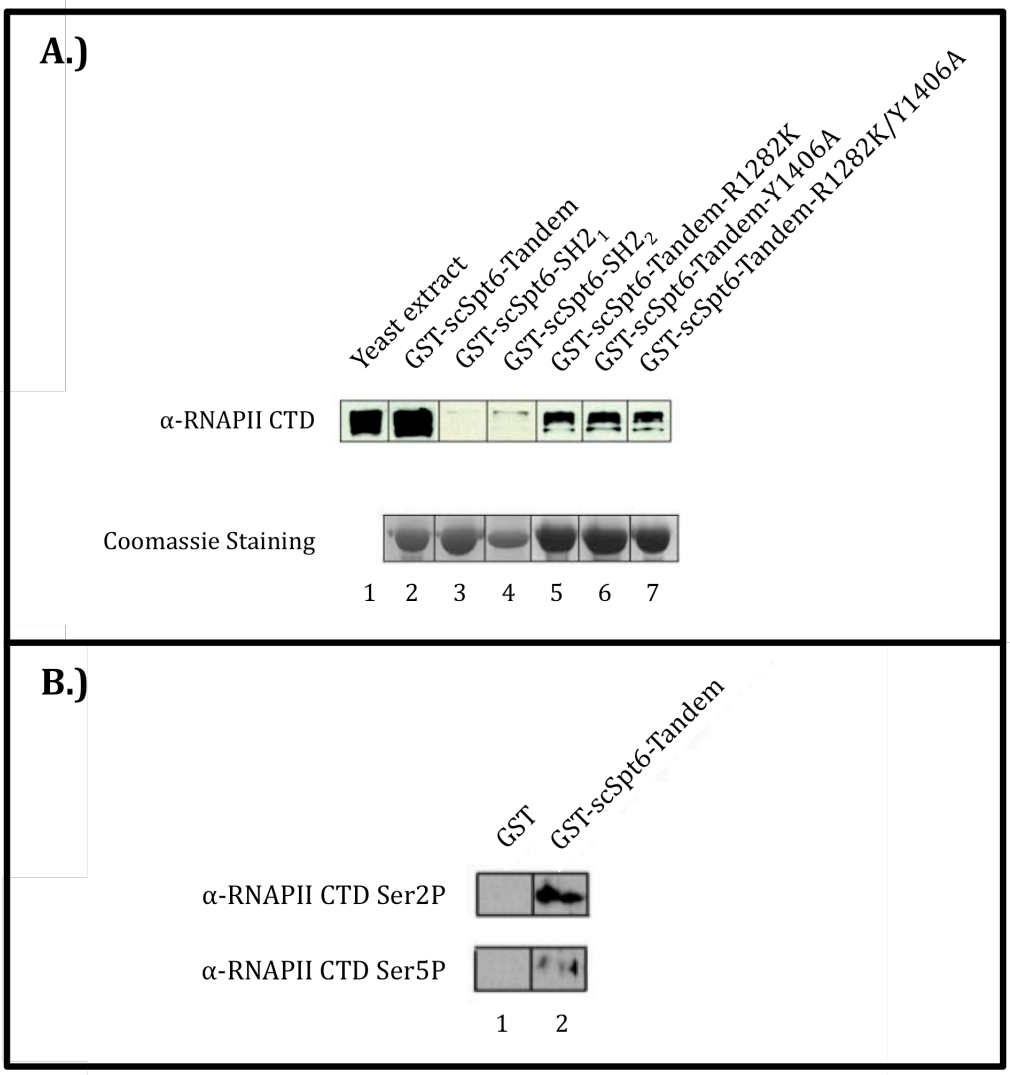

Figure 2-9: Role of SH2 domains in binding the RNAPII CTD and analysis of phosphospecificity of the Spt6 tandem SH2 region (Continued).

residues for SH2 domain function in Spt6 have not yet been identified or multiple changes are necessary to impair SH2 function. Analysis of phosphospecificity of the Spt6 tandem SH2 region shows a slight increase in binding to serine 2-phosphorylated RNAPII CTD compared to serine 5-phoshorylated RNAPII CTD (Figure 2-9B) (Diebold et al., 2010b). In addition, our collaborators solved the structure of the Spt6 tandem SH2 domains in *A. locustae* at 2.2 Å resolution, clearly showing the double SH2 domain structure (Figure 2-10) (Diebold et al., 2010b).

In conclusion, *spt6* mutants lacking a single novel SH2 domain or both SH2 domains have many severe phenotypes; however, Spt6 protein levels are unaffected. Our collaborators solved the structure of the Spt6 C-terminal region and showed that the tandem SH2 domains are necessary and sufficient for binding the RNAPII CTD *in vitro*. Loss of the Spt6-RNAPII CTD interaction appears to drastically impair Spt6 function, most likely by impairing Spt6 recruitment to chromatin and the transcription machinery. This is consistent with recent ChIP-chip studies showing that an *spt6* mutant missing the region encoding the C-terminal 202 amino acids has reduced recruitment over ORFs (Mayer et al., 2010). Interestingly, recruitment of this mutant is not totally abolished, suggesting an RNAPII-CTD-independent mechanism of Spt6 recruitment to chromatin (Mayer et al., 2010). Although the full story is undoubtedly more complex, our results suggest that both SH2 domains modulate the Spt6-RNAPII-CTD interaction, which is crucial for Spt6 function.

Analysis of Spn1-binding Site Mutants

As a second focused analysis of a specific Spt6 domain, I studied the interaction between Spt6 and its conserved binding partner, Spn1/Iws1. This work was also done in

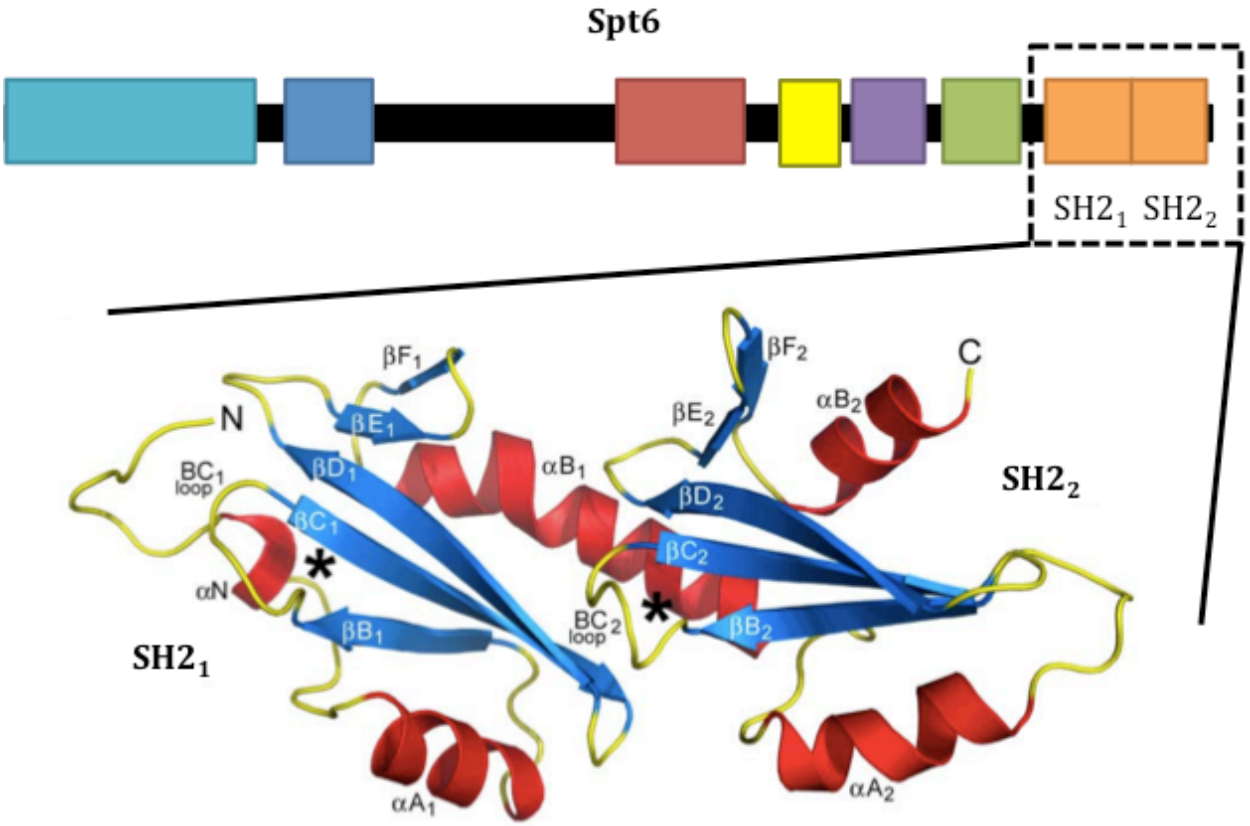

Figure 2-10: Structure of the conserved C-terminal region of Spt6 in *A. locustae*. A ribbon representation of the *A. locustae* Spt6 tandem SH2 structure crystallized by our collaborators at 2.2 Å resolution. α-helices are colored in red, β-strands in blue, and loops in yellow. The putative phosphoresidue-binding pockets of the two SH2 domains are each marked with an asterisk (*). Secondary structural elements are labeled according to the common SH2 nomenclature (Kuriyan et al., 1997). All structural figures were created with PyMOL (Delano Scientific). The portion of Spt6 crystallized is indicated by the dashed box on the cartoon of Spt6 above. The newly discovered SH2 domain is $SH2_2$. (This figure is adapted from Diebold et al., 2010b)

collaboration with Marie-Laure Dibold and Christophe Romier (Diebold et al., 2010a). As previously mentioned, experiments by our collaborators suggested that an N-terminal region of Spt6 (residues 229-269) is necessary and sufficient for Spt6-Spn1 binding *in vitro* in *S. cerevisiae* (Diebold et al., 2010a). Based on this result, our collaborators solved the structure of this N-terminal region of Spt6 bound to Spn1 in *E. cuniculi* at 1.75 Å resolution (Figure 2-11) (Diebold et al., 2010a). Results from other labs were in agreement with ours (McDonald et al., 2010; Pujari et al., 2010). Close examination of this structure suggested a number of Spt6 amino acids in the Spn1-binding pocket that might be required for Spn1 binding and which inspired the experiments described below.

By the methods described in Materials and Methods, I created strains containing *spt6* mutations that were predicted to impair interaction with Spn1: *spt6-YW, spt6-IF,* and *spt6-GG* (Figure 2-12). The *spt6-YW* strain has the largest array of mutant phenotypes and is Spt$^-$, strongly Ts$^-$, HU^s, and sensitive to DNA damaging agents (phleomycin and MMS sensitivity) (Figure 2-13). The *spt6-IF* strain is Spt$^-$ and Ts$^-$ while the *spt6-GG* strain is wild type for all phenotypes tested. Thus, three different double mutants predicted to impair Spt6-Spn1 interactions have a range of phenotypes ranging from severe to non-detectable.

Given the strong phenotype of *spt6-YW*, I decided to further characterize it by examining each point mutation separately. Interestingly, the single point mutants, *spt6-Y* and *spt6-W*, behave differently from *spt6-YW* (Figure 2-13). The *spt6-Y* mutant is weakly Spt$^-$, while *spt6-W* grows poorly on SC-His and SC-Lys plates, referred to here as a "reverse Spt$^-$" phenotype. Therefore, neither of these single point mutants is solely responsible for the phenotype of *spt6-YW*, and the single point mutants behave quite differently alone

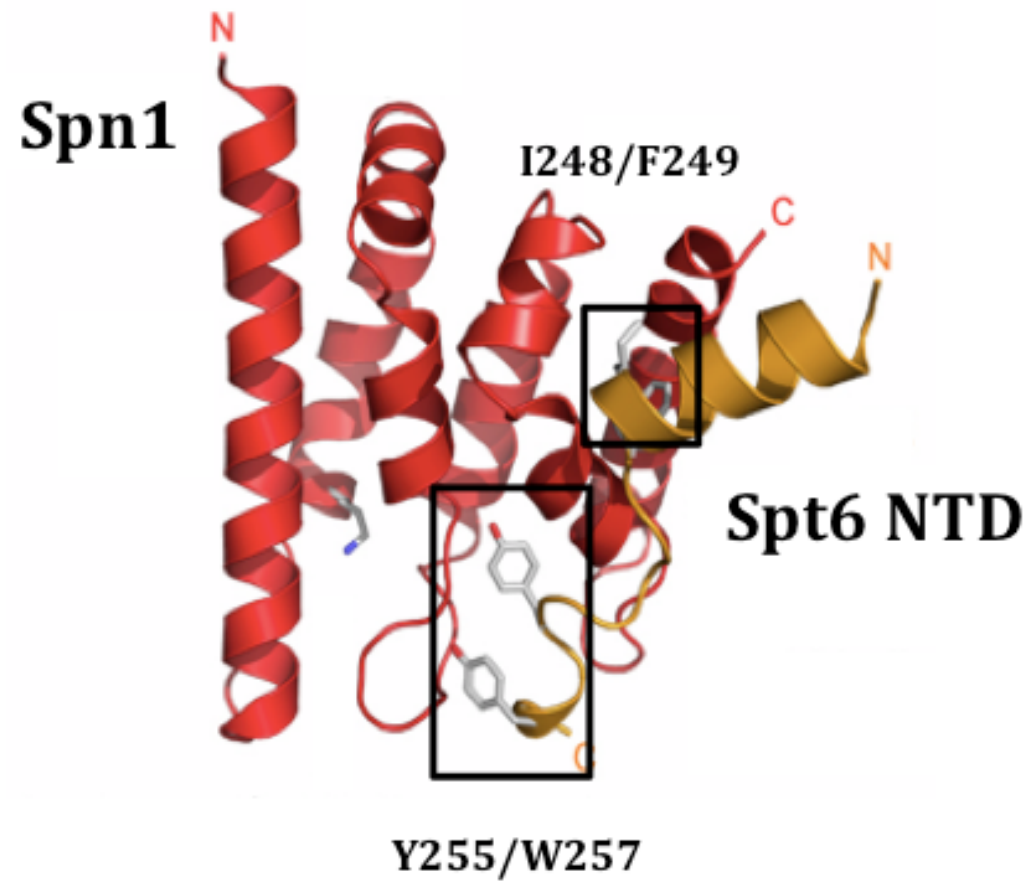

Figure 2-11: Spn1-Spt6 N-terminal region structure in *E. cuniculi*. Ribbon representation of the $Spt6_{34-71}$/$Spn1_{55-198}$ crystal structure solved by our collaborators at 1.75 Å resolution in *E. cuniculi*. All amino acid numbers written as subscripts refer to *E. cuniculi* numbering for the respective proteins. $Spn1_{55-198}$ is the conserved region of *E. cuniculi* Spn1 and $Spt6_{34-71}$ is the region necessary and sufficient for *E. cuniculi* Spn1 binding. Spn1 is shown in red and the N-terminal region of Spt6 is shown in orange. The *E. cuniculi* Spt6 residues corresponding to *S. cerevisiae* amino acids I248/F249 and Y255/W257 are indicated by black boxes. These amino acids are located within the Spt6-Spn1 binding site, which is consistent with their mutant phenotypes. (This figure is adapted from Diebold et al., 2010a)

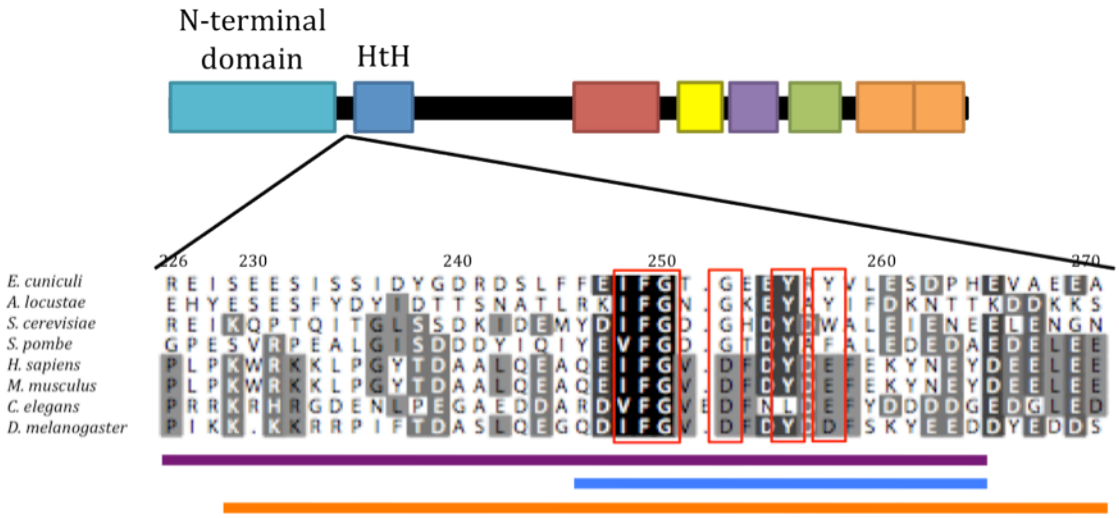

Figure 2-12: Summary of *spt6* mutants that affect Spt6-Spn1 binding in *E. cuniculi*.

The location of the aligned amino acid sequence from Spt6 is shown on the diagram of Spt6 at the top. Darker shading in the sequence alignment correlates with higher conservation. The numbering at the top of the alignment corresponds to the amino acid numbers for *S. cerevisiae*. The *E. cuniculi* Spt6 residues 53-71 (underlined in blue under the sequence alignment) and the *S. cerevisiae* Spt6 residues 229-269 (underlined in orange under the sequence alignment) are necessary and sufficient for Spn1 binding in *E. cuniculi* and *S. cerevisiae*, respectively. The residues changed in the *spt6-IF, spt6-GG*, *spt6-YW, spt6-Y,* and *spt6-W* mutants are highlighted in red. The *E. cuniculi* Spt6 residues crystallized in the structure in Figure 2-11 are underlined in purple under the sequence alignment. (This figure is adapted from Diebold et al., 2010a)

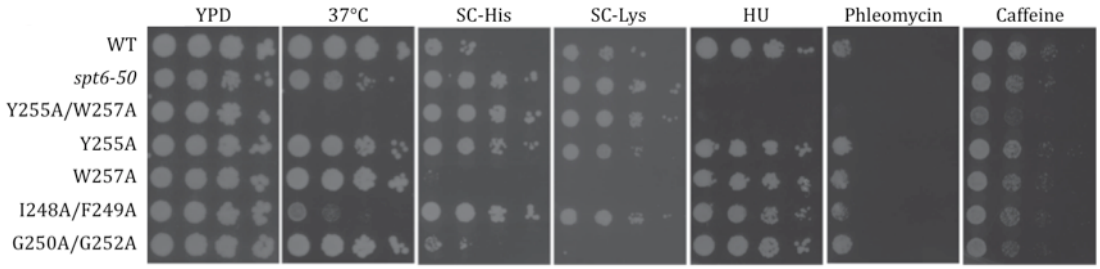

Figure 2-13: Phenotypes of *spt6* mutants with amino acid changes within the Spn1 binding pocket. Each row shows serial dilutions of the indicated yeast strains. The strains used had an *spt6* deletion covered by a *CEN* plasmid containing either FLAG-tagged wild-type (WT) *SPT6* (yEL89), *spt6-50* (yEL90), *spt6-YW* (yEL91), *spt6-Y* (yEL92), *spt6-W* (yEL93), *spt6-IF* (yEL95), or *spt6-GG* (yEL96). The strains were grown to saturation in YPD, serially diluted 10-fold, and spotted on the indicated media. Strain yEL90 (containing *spt6-50*, a well-characterized C-terminal truncation mutant) was used as a positive control. (Abbreviations: Ts^-, temperature sensitivity at 37°C; Spt^-, able to grow on medium lacking lysine (SC-Lys) despite the presence of the *lys2-128δ* insertion allele; HU^S, sensitive to hydroxyurea)

versus when combined. Altogether, these results suggest that residues I248/F249 and Y255/W257 are important for Spt6 function.

To test whether these mutations impair Spt6-Spn1 interactions, I performed co-IP experiments. My results (Figure 2-14) suggest that Spt6-Spn1 binding is decreased in the *spt6-IF*, *spt6-GG*, and *spt6-YW* mutants. For the *spt6-Y* and *spt6-W* mutants, the results were variable over multiple experiments (not shown) and no conclusion about their effect on Spt6-Spn1 binding can be drawn at this time. Interestingly, the *spt6-GG* mutant, which exhibits no mutant phenotypes, appears to impair Spt6-Spn1 binding (discussed below). Overall, these results are consistent with structural data showing the importance of I248/F249, Y255/W257, and G250/G252 in binding to Spn1.

In conclusion, through this collaboration, I have identified a region of Spt6 required for Spt6-Spn1 binding. Mutations in this region affect Spt6 function *in vivo* with *spt6-YW* causing the most severe set of phenotypes. Several of these mutations appear to cause reduced Spt6-Spn1 interaction by co-IP. In addition, our collaborators solved the structure of this Spt6 region bound to Spn1, and our *in vivo* data is overall consistent with this structure. Altogether these experiments nicely indicate the relationship between structure and function in this Spn1-binding N-terminal region of Spt6. This work paves the way for better understanding the complex relationship between Spt6 and Spn1.

Figure 2-14: Co-immunoprecipitation analysis of the effect of point mutations in Spt6 on Spt6-Spn1 binding. Cell lysates were prepared from strains containing either wild-type Spt6, FLAG-tagged Spt6, Spt6-IF, Spt6-GG, Spt6-YW, Spt6-Y, or Spt6-W. After pull-down with α-FLAG antibody coupled to Protein G beads, samples were probed for the presence of Spt6 (α-FLAG antibody) and Spn1 (α-Spn1 antibody, courtesy of Catherine Radebaugh and Laurie Stargell). Input cell lysates and immunoprecipitated (IP) samples are shown. A nonspecific band in the IP is marked by an asterisk (*). The lower band in the IP is Spn1. Spn1 binding is consistently decreased in *spt6-IF, GG,* and *YW*. For the *spt6-Y* and *spt6-W* mutants, the results were variable over multiple experiments (not shown) and no conclusion about their effect on Spt6-Spn1 binding can be drawn at this time. The Spn1 inputs for *spt6-IF* and *spt6-GG* are lower than wild-type controls in this experiment which should be taken into account when interpreting the results.

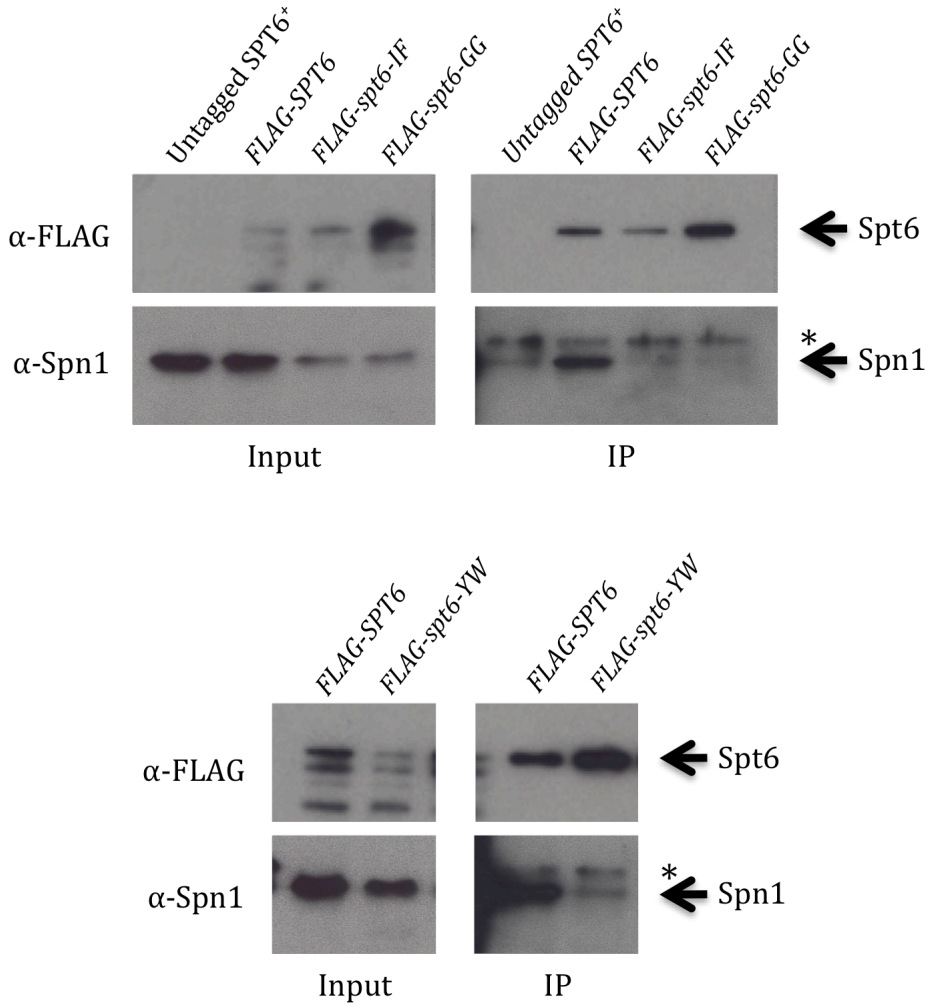

Figure 2-14: Co-immunoprecipitation analysis of the effect of point mutations in Spt6 on Spt6-Spn1 binding (Continued).

Discussion

In this chapter, I characterized three classes of *spt6* mutants. First, I created a library of sixty-seven *spt6* insertion mutants. These insertions are located throughout the *SPT6* coding region and exhibit many diverse phenotypes when over-expressed on plasmids. Surprisingly, when a subset of these mutants were integrated into the genome, replacing the wild-type *SPT6* gene, most of them caused only modest phenotypes despite having insertions in predicted domains of Spt6 (Close et al., 2011; Johnson et al., 2008).

Two of the integrated mutants show strong pleiotropic phenotypes similar to a previously-characterized mutant, *spt6-1004* (Cheung et al., 2008; Kaplan, 2002; Kaplan et al., 2005; Kaplan et al., 2003). This is intriguing given that these two insertion mutants are located in regions encoding the HtH domain and a linker region; different locations than the HhH deletion of *spt6-1004*. These three mutants (*spt6-76, spt6-487,* and *spt6-1004*) all express reduced levels of Spt6 mutant protein; therefore all three may reduce Spt6 levels below a certain threshold level of Spt6 protein that is required for wild-type phenotypes, resulting in an overall loss-of-function phenotype for all three.

A possible explanation for the difference in mutant phenotypes when the *spt6* mutations are on an over-expression plasmid versus integrated into the genome is that increased mutant protein levels cause stronger phenotypes. It has previously been observed that over-expression of wild-type Spt6 protein is sufficient to cause mutant phenotypes such as the Spt$^-$ phenotype (Clark-Adams and Winston, 1987). However, it is unclear what the effect of over-expressing a mutant *spt6* allele would be. Although it is possible that changes in levels of mutant *spt6* protein cause mutant phenotypes, similar to

the wild-type Spt6 protein over-expression scenario, there are currently no examples of this in the Spt6 literature that I am aware of. In the future, it is crucial to integrate these types of mutants into the genomic locus before drawing any conclusions.

During the course of these experiments, a nearly complete structure of Spt6 was published (Close et al., 2011). Interestingly, many of the putative domains previously identified in Spt6 were shown to have structures incompatible with their predicted functions. For example, helix-turn-helix domains normally bind to DNA; however, the HtH domain in Spt6 is not compatible with DNA binding due to the steric clash that would result between the bound DNA molecule and other portions of the Spt6 protein structure (Close et al., 2011). In addition, the Spt6 S1 domain, which is expected to bind nucleic acids, lacks the necessary binding cleft residues and is not required for binding double-stranded DNA (Close et al., 2011). Interestingly, the structural studies identified an Spt6 domain which had been previously overlooked, a death-like domain (DLD). This only added to the mystery of Spt6 since these motifs are usually found in proteins of the inflammatory and apoptotic pathways. Although this study added significant insight into the structure of Spt6, it also highlighted the need for structure-function analysis to understand the function of these domains in the role of Spt6 in chromatin structure and transcription.

In the second part of this chapter, I analyzed two *spt6* mutants encoding C-terminal truncations to address the requirement for the C-terminal tandem SH2 domains. The most significant finding was that both of these mutants have a number of severe mutant phenotypes, although Spt6 protein levels are unaffected. Overall, this suggests that the SH2 domains are important for Spt6 function. This was confirmed by experiments by our

collaborators showing that both SH2 domains are necessary and sufficient for RNAPII CTD binding *in vitro* (Diebold et al., 2010b).

Given that Spt6 had been the only *S. cerevisiae* protein known to have an SH2 domain for many years (Maclennan and Shaw, 1993), the discovery of a second SH2 domain is quite significant (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). Interestingly, the $SH2_2$ domain is fairly divergent from other SH2 domains (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). For example, although the $SH2_2$ domain has a generally conserved secondary structure and domain fold (Kuriyan and Cowburn, 1997), its primary amino acid sequence is divergent from other SH2 domains and its crucial binding residue is changed from the typical arginine to a tyrosine (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). This explains why this SH2 domain was not previously identified by sequence homology analysis. Therefore, structural studies of the Spt6 C-terminal region were crucial to identify this atypical SH2 domain.

In addition, experimentally determined structures of the Spt6 tandem SH2 domains have revealed other unique characteristics of the $SH2_2$ domain. For example, the phosphate-binding pocket is altered in the $SH2_2$ domain with its opening in a different orientation than typically seen (Diebold et al., 2010b). Also, this pocket is occupied by a bulky tyrosine side chain making conventional phosphate-binding unlikely (Close et al., 2011). Therefore, it is hypothesized that $SH2_2$ represents a new SH2 domain subfamily with a unique phosphate-binding site (Liu et al., 2011). It is likely that there are other similar unrecognized SH2 domains in yeast and other organisms, a possibility that should be explored further. In effect, this work has not only doubled the number of known SH2

domains in the *S. cerevisiae* genome from one to two, but has given new insight into the cooperative function of tandem SH2 domains and the divergent structures possible for SH2 domains.

An important unanswered question is what amino acids the $SH2_1$ and $SH2_2$ domains bind and whether these two SH2 domains differ in their preferred target binding residues. Recent work has identified phosphorylation on the tyrosine in the heptapeptide repeats of the RNAPII CTD (Mayer et al., 2012). Interestingly, increased tyrosine phosphorylation in the RNAPII CTD increases interaction of the RNAPII CTD with Spt6 *in vitro* (Mayer et al., 2012). This raises the intriguing possibility that one or both of the Spt6 SH2 domains binds to phosphorylated tyrosine in the RNAPII CTD. Perhaps one SH2 domain primarily binds to phosphorylated serine and the other to phosphorylated tyrosine. However, this is purely conjecture and further experiments will be required to explore this hypothesis.

In my experiments, there was one additional mutant that was not constructed: a deletion of the sequence of *SPT6* encoding just $SH2_1$. Analyzing this mutant would clarify the requirement for $SH2_1$, the functional contribution of each SH2 domain, and indicate whether $SH2_2$ retains any function without its $SH2_1$ counterpart. Future work on a deletion of just $SH2_1$ may yield further helpful insights.

To determine the requirements for specific amino acids in the Spt6 SH2 domains, others have constructed and analyzed several different point mutations within the region encoding the tandem SH2 domains, including double mutants (Close et al., 2011). These *spt6* point mutations include: R1282H, S1284D, R1286A, Q1303E, E1313A/N1314A, or K1343E (all in the $SH2_1$ domain), or P1390A or K1411E (in $SH2_2$). They were screened for a variety of phenotypes including the Spt^- phenotype and sensitivity to high temperature,

hydroxyurea, 6-azauracil, and mycophenolic acid. However, none of these mutants are sensitive to any of the stress conditions tested, relative to the wild-type strain. The one exception was *spt6-Q1303E,* which has an extremely weak Spt$^-$ phenotype (Close et al., 2011). These results indicate that either the crucial residues for SH2 domain function in Spt6 have not yet been identified or multiple changes are necessary to impair SH2 function.

Work by several groups has confirmed the tandem SH2 domain structure of Spt6 (Close et al., 2011; Liu et al., 2011; Sun et al., 2010). A concurrent study published back-to-back with ours also showed that the tandem SH2 domains bind to serine 2-phosphorylated RNAPII CTD peptide *in vitro* (Sun et al., 2010). Also, they noted that deletion of the Spt6 tandem SH2 region in yeast is lethal in the presence of 6-azauracil, indicating that this region is important for transcription elongation. This is consistent with our collaborators' results showing that the SH2 domains are required for binding the RNAPII CTD, particularly serine 2-phosphorylated CTD which is associated with transcription elongation (Diebold et al., 2010b). Also, our analysis shows that SH2 truncation mutants have a strong Spt$^-$ phenotype indicating a defect in transcription regulation. In sum, this work confirms the presence and importance of these tandem SH2 domains.

In the third section of this chapter, I constructed *spt6* point mutants to explore the Spn1-binding site of Spt6. The residues altered in these mutants are located within the Spt6-Spn1 binding pocket, according to the structure of Spn1 binding to an N-terminal section of Spt6 solved by our collaborators (Diebold et al., 2010a). These mutants cause a number of severe mutant phenotypes indicating their functional importance *in vivo*. Consistent with this, co-IP experiments suggested loss of binding between Spt6 and Spn1 in

these mutants. All evidence points to the importance of this N-terminal region of Spt6 for binding Spn1.

One surprising result is that the *spt6-GG* mutant, which has no detectable mutant phenotype, appears to impair Spt6-Spn1 binding by co-IP. The reason for this is unclear. One explanation for this inconsistency is that the loss of the Spt6-Spn1 interaction does not cause a mutant phenotype that I yet know how to detect and that the other mutations, *spt6-IF* and *spt6-YW,* cause mutant phenotypes for a reason independent of their effect on Spt6-Spn1 interactions. Alternatively, there may be a difference between the *in vivo* conditions and the *in vitro* co-IP conditions that might be misleading, at least in the case of *spt6-GG;* by this explanation, Spt6-Spn1 interactions are only impaired *in vivo* in the *spt6-YW* and *spt6-IF* mutants, and not in the *spt6-GG* mutant. Regardless of the reason, in light of this inconsistency, the co-IP experiments must be interpreted with caution.

Two additional groups have published structures of Spn1 and/or Spn1 bound to a portion of Spt6 (McDonald et al., 2010; Pujari et al., 2010) and these structures correspond well to the structure solved by our collaborators. Work from the Formosa and Hill labs confirmed the region of Spt6 required for Spn1 binding (McDonald et al., 2010). Intriguingly, they also showed by gel shift assay that this same Spt6 region is also required for nucleosome binding and their results suggest that Spn1 and nucleosome binding to Spt6 are mutually exclusive. This suggests a model whereby Spn1 disengages Spt6 from nucleosomes to allow multiple rounds of nucleosome reassembly. Another possible model is that Spn1 modulates the equilibrium between the involvement of Spt6 in nucleosome reassembly and in mRNA processing. Either way, it is clear that Spn1 plays an important role in the interaction of Spt6 with nucleosomes.

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Chapter 3

Exploring a Role for Spt6 in Transcription-associated Mutagenesis

Attribution of Experiments in Chapter 3

All experiments and figures were completed by Erin Loeliger.

Abstract

Spt6, a key factor involved in regulating chromatin structure, is conserved throughout eukaryotes. Spt6 has been shown to be required for a number of functions, including nucleosome assembly, transcription initiation and elongation, and mRNA processing and export. Interestingly, previous preliminary work has also implicated Spt6 in DNA damage repair. However, this area of Spt6 biology has been explored very little. A tantalizing hypothesis is that Spt6 serves to facilitate DNA damage repair during transcription. To address this possibility, I have attempted to test for a possible role for Spt6 in transcription-associated mutagenesis. After employing several types of *in vivo* assays, I conclude that a possible role for Spt6 in transcription-associated mutagenesis is uncertain, as the results (with respect to a role for Spt6) reproducibly vary depending on the assay used. Thus, understanding this aspect of Spt6 biology awaits better assays and understanding of transcription-associated mutagenesis.

Introduction

As discussed in Chapter 1, chromatin structure is an obstacle to DNA repair (Gospodinov and Herceg, 2013; Huertas et al., 2009; Seeber et al., 2013; Tsabar and Haber, 2013; Tsukuda et al., 2009). Recent work indicates that changes in histone structure and modifications are required to recruit factors necessary for DNA damage repair (Groth et al., 2007; Tsukuda et al., 2009). A growing number of histone chaperones and chromatin remodeling complexes have been implicated in this process, including Arp4, Hif1, CAF-1, Asf1, DNMT1, Rad54, and the ISW1a, ISW1b, ISW2, CHD1, FUN30, INO80, SWR1, RSC, and Swi/Snf complexes (De Koning et al., 2007; Gospodinov and Herceg, 2013; Groth et al., 2007; Kim and Haber, 2009; Seeber et al., 2013; Tsabar and Haber, 2013).

The role of Spt6 in chromatin structure raises the intriguing possibility that Spt6 may also play a role in DNA repair (Bortvin and Winston, 1996; Degennaro et al., 2013; Kaplan et al., 2003). Indeed, previous studies (I. Ivanovska, unpublished data) as well as work in Chapter 2 have uncovered *spt6* mutants that are sensitive to phleomycin, indicating defects in DNA damage repair. In addition, *spt6* mutants interact genetically with a deletion of *DUN1*, a component of the main double-strand break repair pathway in *S. cerevisiae* (I. Ivanovska, unpublished data). The goal of this chapter was to explore a potential role for Spt6 in DNA damage repair. For this chapter, I will be focusing on a possible role for Spt6 in transcription-associated mutagenesis. (Experiments to test for a role of Spt6 in double-strand break repair are presented in Appendix 1.)

This project arose from an unexpected observation based on nucleosome occupancy data for chromosome III in an *spt6* mutant, *spt6-1004* (Ivanovska et al., 2011). In an *spt6-*

1004 mutant, nucleosomes are lost from highly transcribed genes, suggesting that one role of Spt6 is to replace nucleosomes in the wake of transcription (Degennaro et al., 2013; Ivanovska et al., 2011; van Bakel et al., 2013). However, this nucleosome loss does not correlate with Spt6-mediated changes in mRNA levels or cryptic initiation. For example, in *spt6* mutants, there are genes with significant nucleosome loss but no change in steady-state mRNA levels (Ivanovska et al., 2011). There are also genes with cryptic initiation but no significant nucleosome loss in at *spt6* mutant (Ivanovska et al., 2011). These results suggest that the effect of Spt6 on chromatin structure is independent of its effect on transcription. The intriguing unanswered question is why is it important for nucleosomes to be replaced over highly transcribed coding regions if transcription and cryptic initiation are not affected. One hypothesis is that the histone chaperone activity of Spt6 is important for preventing transcription-associated mutagenesis or TAM.

TAM refers to the observation that increased transcription of a gene leads to higher levels of mutagenesis across the gene (Lippert et al., 2011; Mischo et al., 2011; Takahashi et al., 2011). This has been seen in bacteriophage T7, *E. coli*, *S. cerevisiae*, and mammalian cells (Beletskii and Bhagwat, 1996; Beletskii et al., 2000; Datta and Jinks-Robertson, 1995; Hendriks et al., 2010; Hendriks et al., 2008; Klapacz and Bhagwat, 2002). In yeast, induction of transcription with a strong promoter increases mutagenesis between 7-fold to 30-fold, depending on the promoter (Lippert et al., 2011; Takahashi et al., 2011). While TAM appears to increase many classes of mutations, elevations in 2-3 bp deletions are particularly characteristic (Lippert et al., 2011; Takahashi et al., 2011). The mechanism of TAM is unclear: however, one explanation is that open, transcriptionally-active chromatin is more sensitive to DNA damage (Lippert et al., 2011; Svejstrup, 2010).

This possibility fits well with the role of Spt6 as a histone chaperone, restoring chromatin following transcription and protecting from mutagenesis. I have therefore performed experiments to test whether Spt6 has an effect on transcription-associated mutagenesis. Based on results from several different *in vivo* assays, I conclude that a possible role for Spt6 in transcription-associated mutagenesis is uncertain, as the results reproducibly vary depending on the assay used. The development of better assays and understanding of transcription-associated mutagenesis is required to further study this aspect of Spt6 biology.

Materials and Methods

Strain Construction

URA3 mutagenesis assay: Strains for the *URA3* mutagenesis assay were derived from strain FY1856 (Table 3-1). The *spt6-1004* mutation was introduced using the integrating plasmid pCK25 (contains FLAG-*spt6-1004* in *URA3*-marked pRS306) (Sikorski and Hieter, 1989). In both the wild-type and *spt6-1004* strains, the open reading frame (ORF) of one of four different non-essential genes (*CHA1*, *GLK1*, *BUD3*, or *VAC17*) was replaced with the *URA3* ORF to create the strains yEL389, yEL391, yEL393, yEL395, yEL396, yEL398, yEL400, and yEL402 (Table 3-1). The primers used are shown in Table 3-2.

pGAL1-CAN1 mutagenesis assay: Strains containing *pGAL1-CAN1* and deletion of the *GAL80* gene (*gal80Δ*) were kindly supplied by Sue Jinks-Robertson's lab (Lippert et al., 2011). The *spt6-1004* mutation was introduced using the integrating plasmid pCK25.

Table 3-1: Yeast Strains Used In This Chapter.

Strain	Purpose	Genotype
FY1856	Mutagenesis assay strain construction	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0*
yEL389	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 cha1Δ::URA3*
yEL391	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 cha1Δ::URA3 spt6-1004*
yEL393	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 glk1Δ::URA3*
yEL395	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 glk1Δ::URA3 spt6-1004*
yEL396	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 bud3Δ::URA3*
yEL398	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 bud3Δ::URA3 spt6-1004*
yEL400	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 vac17Δ::URA3*
yEL402	*URA3* mutagenesis assay, Northern blot analysis	*MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 vac17Δ::URA3 spt6-1004*
yEL462	*pGAL1-CAN1* mutagenesis assay	*MATα his3Δ200 ura3ΔNco ade2-101oc suc2 gal80Δ::HIS3*
yEL463	*pGAL1-CAN1* mutagenesis assay	*MATα his3Δ200 ura3ΔNco ade2-101oc suc2 gal80Δ::HIS3 spt6-1004*
yEL464	*pGAL1-CAN1* mutagenesis assay	*MATα his3Δ200 ura3ΔNco ade2-101oc suc2 gal80Δ::HIS3 kanMX6/pGAL-CAN1*
yEL465	*pGAL1-CAN1* mutagenesis assay	*MATα his3Δ200 ura3ΔNco ade2-101oc suc2 gal80Δ::HIS3 kanMX6/pGAL-CAN1 spt6-1004*

Table 3-1: Yeast Strains Used In This Chapter (Continued).

Strain	Purpose	Genotype
yEL473	*pTET-LYS2* mutagenesis assay	*MAT***a** *ura3-52 ade2-101oc trp1Δ1 lys2Δ::hyg leu2-K:TetR-Ssn6:LEU his4Δ::LYS2 his4Δ::lys2ΔA746NR,(AT)2*
yEL474	*pTET-LYS2* mutagenesis assay	*MAT***a** *ura3-52 ade2-101oc trp1Δ1 lys2Δ::hyg leu2-K:TetR-Ssn6:LEU his4Δ::LYS2 his4Δ::lys2ΔA746NR,(AT)2 spt6-1004*
yEL476	*pTET-LYS2* mutagenesis assay	*MAT***a** *ura3-52 ade2-101oc trp1Δ1 lys2Δ::hyg leu2-K:TetR-Ssn6:LEU his4Δ::LYS2 his4Δ::pTET-LYS2 his4Δ::pTET-lys2ΔA746NR,(AT)2*
yEL477	*pTET-LYS2* mutagenesis assay	*MAT***a** *ura3-52 ade2-101oc trp1Δ1 lys2Δ::hyg leu2-K:TetR-Ssn6:LEU his4Δ::LYS2 his4Δ::pTET-LYS2 his4Δ::pTET-lys2ΔA746NR,(AT)2 spt6-1004*

Table 3-2: Primers Used In This Chapter.

Primer	Purpose	Sequence (5' to 3')
ELO59	Use with ELO60 to replace *CHA1* ORF with *URA3* ORF - 5'	agtgctggatagacaagagacaggaaaatt aaccagcgagatgtcgaaagctacatataa
ELO60	Use with ELO59 to replace *CHA1* ORF with *URA3* ORF - 3'	aagggcaaattgatgcttcaacgaaaaagtt attggattttagttttgctggccgcatct
ELO63	Use with ELO64 to replace *GLK1* ORF with *URA3* ORF - 5'	accacaacaccaccactaatacaactctatca tacacaagatgtcgaaagctacatataa
ELO64	Use with ELO63 to replace *GLK1* ORF with *URA3* ORF - 3'	acggtgggatacgtacacaaaccaaaaaaatg taaaaagatagttttgctggccgcatct
ELO67	Use with ELO68 to replace *BUD3* ORF with *URA3* ORF - 5'	tgttatctggttgctaaaagagtatatttacacct caccaatgtcgaaagctacatataa
ELO68	Use with ELO67 to replace *BUD3* ORF with *URA3* ORF - 3'	ttgcattaaattaaaaagaaaaaaaaaatcaata aaacactagttttgctggccgcatct
ELO73	Use with ELO74 to replace *VAC17* ORF with *URA3* ORF - 5'	aagaaacagctcgcataaggaaacaaggacaca tcgattaatgtcgaaagctacatataa
ELO74	Use with ELO73 to replace *VAC17* ORF with *URA3* ORF - 3'	tggagcaaaagaagagtaggttaggtaaaggaggc attaatagttttgctggccgcatct
FO1324	Use with FO1325 to amplify 190 bp of *SNR190* ORF as template for Northern blot probe - 5'	ggccctgatgataatg
FO1325	Use with FO1324 to amplify 190 bp of *SNR190* ORF as template for Northern blot probe - 3'	ggctcagatctgcatg

Table 3-2: Primers Used In This Chapter (Continued).

Primer	Purpose	Sequence (5' to 3')
FO3672	Use with FO3673 to amplify 175 bp of *URA3* ORF as template for Northern blot probe - 5'	gcaagggctccctagctact
FO3673	Use with FO3672 to amplify 175 bp of *URA3* ORF as template for Northern blot probe - 3'	aatgcgtctcccttgtcatc

The strains used for this mutagenesis assay were yEL462, yEL463, yEL464 and yEL465 (Table 3-1).

pTET-LYS2 mutagenesis assay: Strains containing *pTET-LYS2* were kindly supplied by Sue Jinks-Robertson's lab (Lippert et al., 2011). The *spt6-1004* mutation was introduced using the integrating plasmid pCK25. The strains used for this mutagenesis assay were yEL473, yEL474, yEL476, and yEL477 (Table 3-1).

Growth of Strains for TAM Analysis

The mutagenesis assay protocols were optimized with respect to starting cell dilution (10^3 cells/ml or 10^6 cells/ml), growth time until heat shock (3 hours or 5 hours), length of heat shock (80, 100, 120, 150 minutes, 3.5, 8, 24, or 48 hours), temperature of heat shock (30, 32, 34, 36, or 37°C) and length of grow-out after heat shock (0, 5, 12, 22, 30, 36, 50, or 72 hours). In the optimized protocol, cultures derived from single yeast colonies were grown until saturated and then diluted back to 10^3 cells/ml. After five hours of growth at 30°C, half of the cultures were shifted to 37°C for 80 minutes to inactivate the temperature-sensitive Spt6-1004 protein. Cells were then returned to 30°C and grown to saturation for two to three days. For the *URA3*-based mutagenesis assay, cells were plated on 5-fluoroorotic acid (5-FOA) (to detect mutations in *URA3*) and on rich medium (to measure cell viability). The number of colonies for each strain and condition were counted and used to calculate the mutation frequency.

For the other mutagenesis assays, a similar protocol was used. For the *pGAL1-CAN1* system, cells were grown in YPGE medium (rich medium with ethanol and glycerol as the carbon source). Cells were then plated on canavanine (to detect mutations in *CAN1*) and on

rich medium (to measure cell viability). Lastly, for the *pTET-LYS2* system, cells were grown in rich medium. Cells were then plated on medium lacking lysine (SC-Lys, to detect mutations in a mutant *lys2* allele containing a frameshift mutation) and on rich medium (to measure cell viability).

Northern Blot Analysis

RNA extraction and Northern hybridization experiments were performed as previously described (Ausubel et al., 1987). Strains were grown under the same conditions used for the TAM assays. Northern blot analysis was performed with probes to the ORFs of *URA3* (+464 to +638, where +1 is the ATG), and the loading control, *SNR190* (+1 to +190). The primers used are listed in Table 3-2.

Results

Analysis of the Role of Spt6 in TAM Using a *URA3*-based Mutagenesis Assay

As a first step to determine whether Spt6 is involved in TAM, we developed a mutagenesis assay using the *URA3* gene as a reporter (Figure 3-1A). Wild-type and *spt6-1004* strains were constructed with the *URA3* ORF replacing the ORF of one of four different non-essential genes on chromosome III: *CHA1*, *GLK1*, *BUD3*, or *VAC17*. *CHA1* and *GLK1* are both highly transcribed genes that experience high nucleosome loss in an *spt6-1004* mutant, while *BUD3* and *VAC17* are both lowly transcribed genes that undergo little nucleosome loss in an *spt6-1004* mutant (Ivanovska et al., 2011). Based on promoter-swapping experiments (I. Ivanovska, unpublished data), the promoters for these genes

Figure 3-1: Mutations in *spt6* appear to cause increased transcription-associated mutagenesis at some loci. A. Strategy for the *URA3* reporter system for detecting TAM. The *URA3* ORF was inserted into either a wild-type strain or an *spt6-1004* mutant, replacing the ORF of the endogenous gene. Increased mutagenesis in *URA3* would result in a higher frequency of colonies with non-functional *URA3* and, therefore, that have become resistant to 5-FOA. **B.** Measurement of the frequency of 5-FOA^R colonies in *SPT6* (wt) and *spt6-1004* strains grown at 30°C or with a shift to 37°C, following the protocol described in Materials and Methods. Each value represents the average of results from four separate experiments for the *GLK1* and *VAC17* strains and ten separate experiments for the *CHA1* and *BUD3* strains. The error bars show the standard error. (Abbreviation: cfu, colony-forming unit)

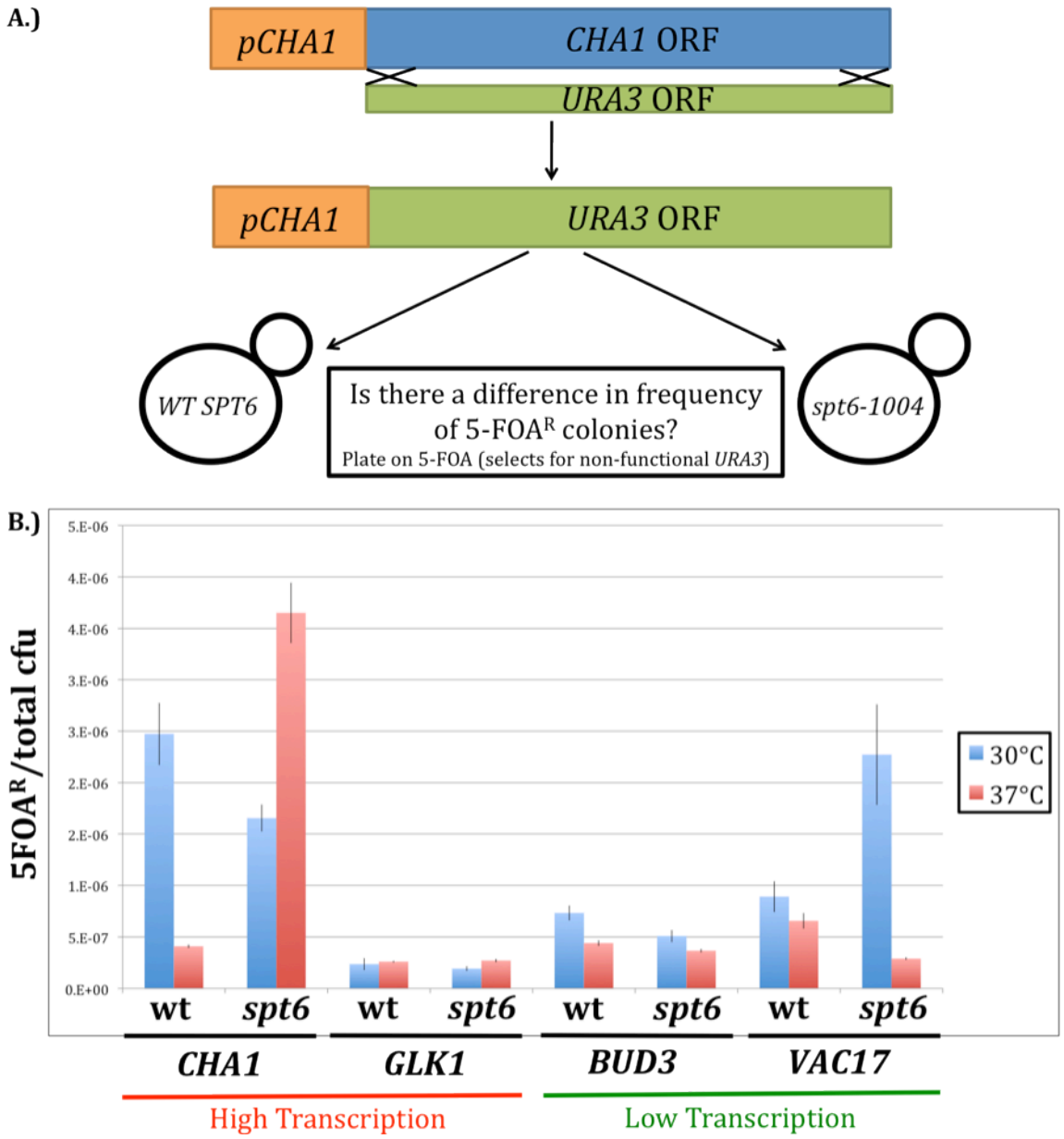

Figure 3-1: Mutations in *spt6* appear to cause increased transcription-associated mutagenesis at some loci (Continued).

appear to be the main determinant for nucleosome loss over the ORF. An important assumption in this experiment is that, given that the promoters of these genes determine nucleosome occupancy, the degree of nucleosome loss will be similar over the *URA3* ORF as over the endogenous gene.

If Spt6 is required for preventing TAM, I expected the *CHA1::URA3* and *GLK1::URA3* constructs to have increased mutagenesis in an *spt6-1004* mutant compared to wild type, particularly after a shift to 37°C, the non-permissive temperature for *spt6-1004*. Our hypothesis also predicted that *BUD3::URA3* and *VAC17::URA3* would show little or no increase in mutagenesis in an *spt6-1004* mutant.

While some results supported our hypothesis, others did not. First, it should be noted that all mutagenesis assays were highly reproducible, as indicated by the error bars associated with the data. The most interesting results were observed in the samples (shown in red) which had undergone a shift to 37°C to reduce Spt6 function (Figure 3-1B). As predicted, we saw a significantly higher level of mutagenesis in the *CHA1::URA3 spt6-1004* strain that had undergone a temperature shift. However, the highly expressed *GLK1::URA3* construct behaved differently than *CHA1::URA3* in an *spt6-1004* mutant, showing low levels of mutagenesis equivalent to wild type. As expected, the lowly transcribed *BUD3::URA3* and *VAC17::URA3* constructs both showed a low mutagenesis level in the wild-type and *spt6-1004* background. Overall, results from this assay following heat shock suggest that the *spt6-1004* mutation increases transcription-associated mutagenesis at some but not all highly-transcribed genes.

In addition, results from the strains that had not undergone heat shock (shown in blue) were equally unexpected (Figure 3-1B). For *CHA1::URA3* strains which were not exposed to heat shock, TAM was high in both wild-type and *spt6-1004* backgrounds with a small decrease in *spt6-1004*. All other TAM values for the strains that had not experienced heat shock were low, except for those for the *VAC17::URA3 spt6-1004* strain. Although the reason for these results is unclear, it shows that different outcomes occur at 30°C versus 37°C. This could be due to involvement of the heat shock response, which may affect TAM. Overall, these results suggest that the role of Spt6 in TAM is more complex than expected.

To test if the different effects at *CHA1* and *GLK1* might be due to different transcription levels, I performed a Northern blot analysis of *URA3* mRNA levels in these strains (Figure 3-2). Please note that this experiment was done only one time so the results must be considered as preliminary. This experiment suggests that there are much higher *URA3* mRNA levels at *CHA1::URA3* compared to *GLK1::URA3*, possibly explaining the differences observed between *CHA1::URA3* and *GLK1::URA3* in the mutagenesis assay. *URA3* mRNA levels were low at *BUD3::URA3* and *VAC17::URA3* as expected.

To summarize the results from the *URA3* TAM assay, after a temperature shift of an *spt6-1004* mutant to inactivate Spt6, I observed a 9-fold increase in the level of mutations in the highly transcribed gene *CHA1::URA3* construct. However, this effect was not observed at *GLK1::URA3.* Based on a preliminary Northern blot experiment, this discrepancy may be explained by mRNA transcription levels which are much higher for *CHA1::URA3* compared to *GLK1::URA3*. TAM levels were low at the *BUD3* and *VAC17* loci as expected. Therefore, TAM reporters at three out of the four genes examined behaved as would be expected if Spt6 is required for preventing TAM.

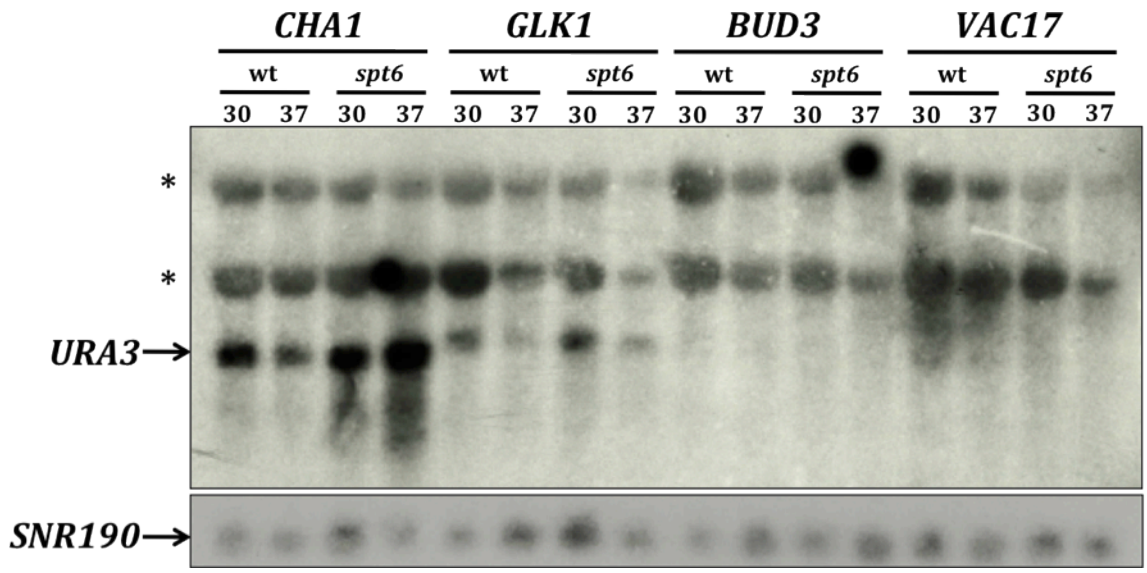

Figure 3-2: Preliminary Northern blot analysis for *URA3* transcript levels in the *URA3* TAM assay. Preliminary Northern blot of the strains used for the *URA3* TAM assay. *SNR190* was used as a loading control. As can be seen in the *SNR190* panel, the overall mRNA loading is slightly uneven and should be taken into account when making conclusions regarding the *URA3* transcript levels. It should be noted that this experiment was only done once. The two upper bands marked by asterisks (*) are caused by non-specific binding of the *URA3* probe to 25S and 18S rRNA which are highly abundant in RNA preps.

Analysis of the Role of Spt6 in TAM Using a *CAN1*-based Mutagenesis Assay

To test further for a role for Spt6 in TAM, I turned to TAM assays that were well-established in the literature (Lippert et al., 2011; Mischo et al., 2011; Takahashi et al., 2011). These assays employ *CAN1* or *LYS2*-based reporters to detect effects on TAM. While the *URA3* mutagenesis system is useful because it employs loci where the nucleosome loss patterns in an *spt6* mutant are known, these alternative systems are better-established. They are also likely to be more sensitive given 1) the huge change in transcription that can be attained with inducible promoters, and 2) the large magnitude of TAM effects seen with these assays (20-fold to 70-fold compared to 9-fold for the *URA3* assay) (Lippert et al., 2011; Mischo et al., 2011; Takahashi et al., 2011). Using several different mutagenesis assays would enable me to examine the possible role of Spt6 in TAM more comprehensively.

Previous studies have used the *CAN1* gene under the control of an inducible promoter (either galactose or tetracycline-inducible) to determine mutagenesis rates under high and low transcription conditions (Lippert et al., 2011; Takahashi et al., 2011). The read-out for mutagenesis in this system is the number of canavanine-resistant colonies, as a result of mutations in *CAN1*. To use this type of system, I replaced the wild-type *SPT6* gene with the *spt6-1004* mutant in these strains in order to assay its effect on TAM, as measured by this system.

The *CAN1*-based system I used employed a galactose-inducible promoter to control *CAN1* transcription (referred to here as *pGAL1-CAN1*) (Figure 3-3A) (Lippert et al., 2011). In the control strain (*pCAN1-CAN1*), *CAN1* is transcribed at a low level under the control of its endogenous promoter. In the high expression strain (*pGAL1-CAN1*), *CAN1* is under the

Figure 3-3: Effect of *spt6-1004* on the *pGAL1-CAN1* TAM assay. A. Strategy for the *pGAL1-CAN1* reporter system for detecting TAM. In this system, transcription of the *CAN1* ORF is controlled either by its endogenous promoter (low transcription) or by the *GAL1* promoter (high transcription). The *GAL80* gene is deleted to allow for constitutive expression of the *GAL1* promoter. Increased mutagenesis of *CAN1* will result in a higher frequency of Can^R colonies. (Lippert et al., 2011; Takahashi et al., 2011) **B.** Measurement of the frequency of Can^R colonies in *SPT6* (wt) and *spt6-1004* strains grown at 30°C or with a shift to 37°C, following the protocol described in Materials and Methods. Each value represents the average of results from two separate experiments and therefore no error bars are shown. (Abbreviation: cfu, colony-forming unit)

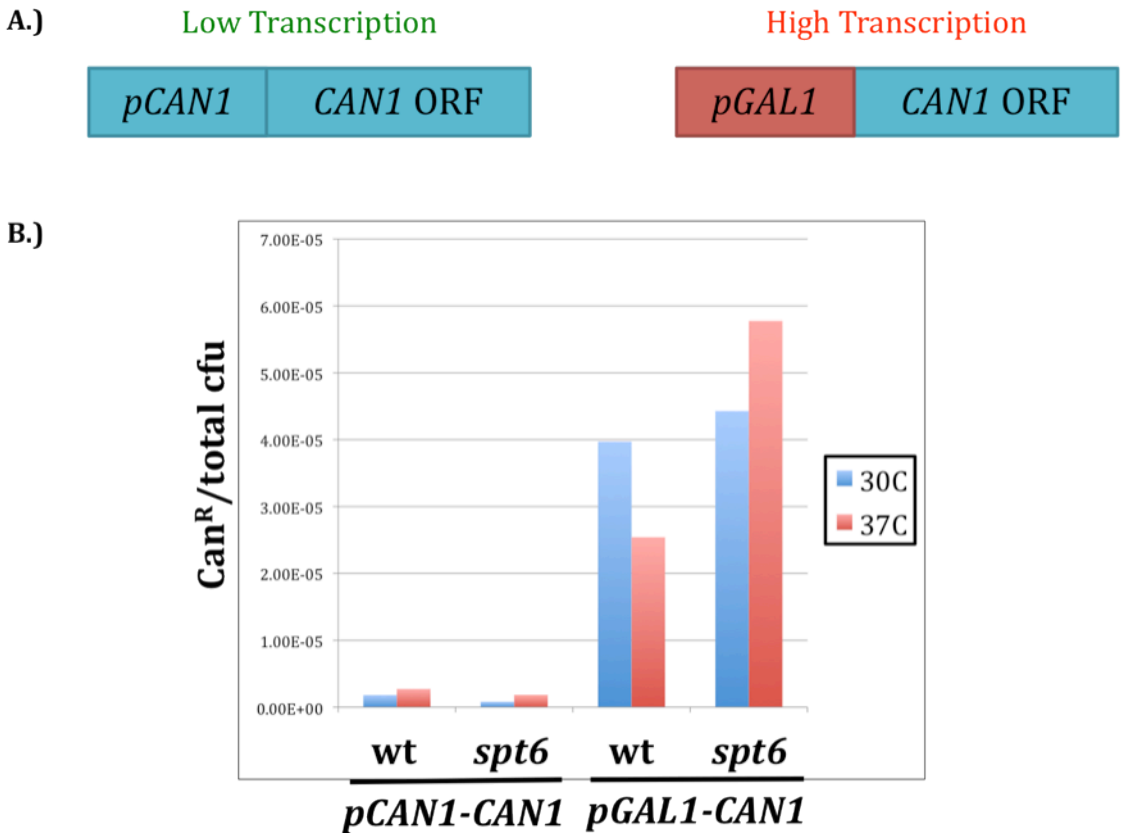

Figure 3-3: Effect of *spt6-1004* on the *pGAL1-CAN1* TAM assay (Continued).

control of the galactose-inducible promoter. The gene encoding the Gal80 repressor is deleted in this strain background, causing *pGAL1-CAN1* to be constitutively expressed (Datta and Jinks-Robertson, 1995).

I examined the effect of an *spt6* mutation on both of these strains. With the wild-type *SPT6* gene present, the results reproduced what had been previously published: mutagenesis increases with transcription (Figure 3-3B). In the *spt6-1004* mutant background, as expected, there was no effect on the *pCAN1-CAN1* strain. In the *spt6-1004 pGAL1-CAN1* strain, there was a modest (2-fold) increase in mutagenesis after a 37°C temperature shift compared to the wild-type *pGAL1-CAN1* strain. This suggests that *spt6-1004* causes a modest increase in TAM by this assay.

As also noted for the *URA3*-based assay, a 37°C temperature shift appears to decrease TAM in wild-type strains. The reason for this is unclear. Perhaps higher temperatures trigger a change in transcription levels or induce a protective response in functional wild-type strains which decreases mutagenesis.

It also should be noted that previous work has shown that *spt6-1004* impairs activation of galactose-inducible promoters (Kaplan et al., 2005). Kaplan et al demonstrated that, compared to wild-type strains, the *spt6-1004* mutant has slower induction kinetics as well as lower steady-state levels (~40-50%) of galactose-induced transcripts (Kaplan et al., 2005). Therefore, it is possible that *spt6-1004* is sabotaging the galactose-inducible system itself and masking a greater effect on TAM.

Analysis of the Role of Spt6 in TAM Using a *LYS2*-based Mutagenesis Assay

Finally, I employed a mutagenesis assay based on *LYS2* (Figure 3-4A) (Lippert et al., 2011). This system contains a frameshift mutation in the *LYS2* ORF that prevents cell growth on medium lacking lysine (SC-Lys). Reversion mutations that correct this frameshift are detected by screening for Lys^+ colonies. To determine if mutagenesis is linked to transcription, expression of *LYS2* is controlled by a tetracycline-inducible promoter.

In a wild-type *SPT6* background, the mutagenesis assay behaved as previously described, with greater mutagenesis associated with higher transcription (Figure 3-4B). Surprisingly, I observed greatly decreased mutagenesis in the presence of *spt6-1004*, the opposite of what would have been expected based on the *URA3* and *pGAL1-CAN1* assays. Based on preliminary Northern blot and quantitative real time PCR (qPCR) results (data not shown), it is very likely that *spt6-1004* is affecting the tetracycline-inducible promoter used here, causing reduced *LYS2* induction and confounding this assay. This emphasizes the limitations and variability of the current TAM assays and the need for newer TAM assays to be developed.

Summary

The overall results of all TAM assays are summarized in Table 3-3. All assays were reproducible in my hands, and I was able to reproduce the effects shown previously in the literature. However, the effect of *spt6-1004* on these assays is confusing. Results from the *URA3* and *pGAL1-CAN1* systems suggest that *spt6-1004* increases TAM, while the *pTET-LYS2* system shows decreased TAM in an *spt6-1004* mutant strain. Furthermore, it

Figure 3-4: Effect of *spt6-1004* on the *pTET-LYS2* TAM assay. A. Strategy for the *pTET-LYS2* reporter system for detecting TAM. In this system, transcription of the *LYS2* ORF is controlled either by its endogenous promoter (low transcription) or by the *pTET-off* promoter (high transcription in the absence of doxycycline). A frameshift mutation is present within the *LYS2* ORF (*) that prevents the cell from growing on medium lacking lysine (SC-Lys). Increased mutagenesis of *lys2** will result in a higher frequency of reversion back to a wild-type functional *LYS2* allele, generating Lys^+ colonies. (Lippert et al, 2011) **B.** Measurement of the frequency of Lys^+ colonies in *SPT6* (wt) and *spt6-1004* strains grown at 30°C or with a shift to 37°C, following the protocol described in Materials and Methods. Each value represents the average of results from two separate experiments and therefore no error bars are shown. (Abbreviation: cfu, colony-forming unit)

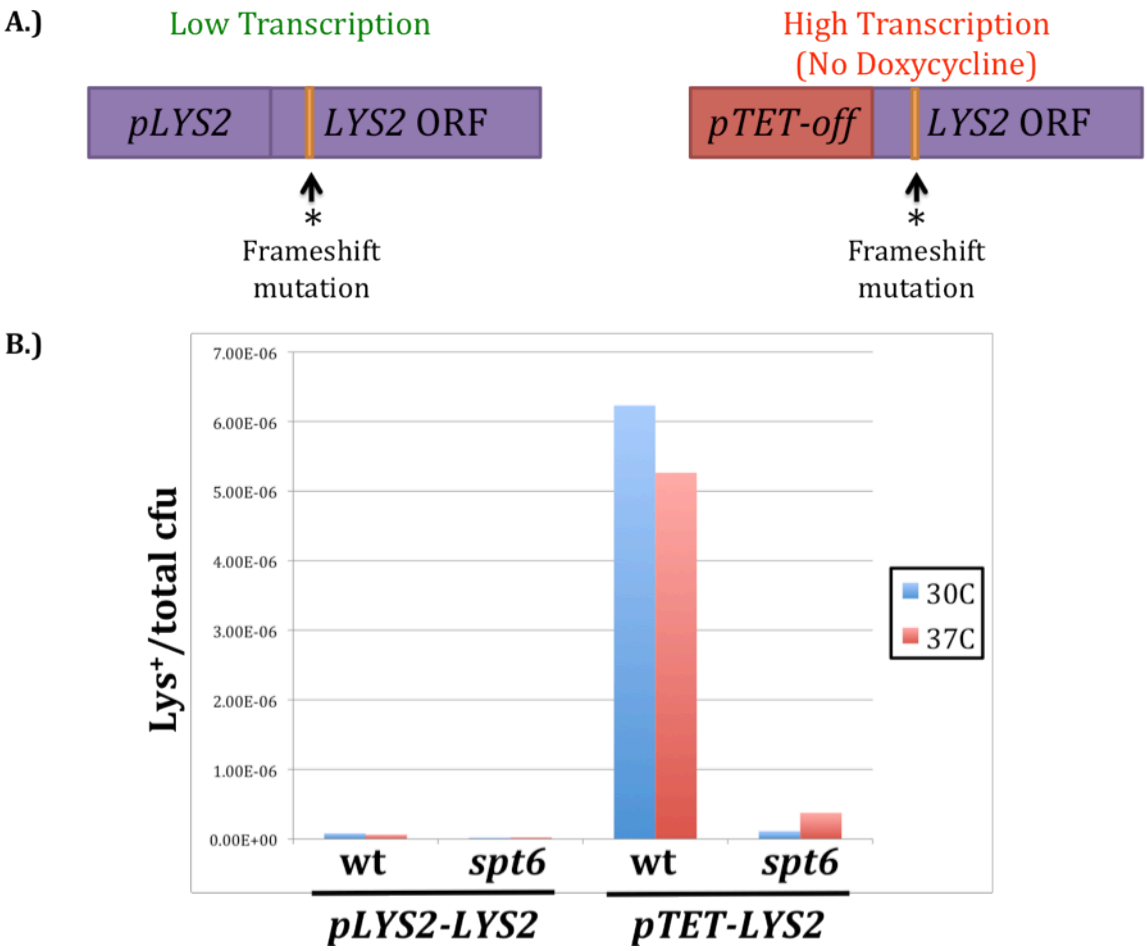

Figure 3-4: Effect of *spt6-1004* on the *pTET-LYS2* TAM assay (Continued).

Table 3-3: Overview of TAM Assay Results.

TAM Assay	Reproducibility	Consistent with literature?	Effect of *spt6-1004* on TAM	Effect of 37°C on TAM	Caveats
URA3	Yes (10/10)	Not applicable	Increased at *CHA1* at 37°C; other genes unaffected	Inconsistent	
pGAL1-CAN1	Yes (2/2)	Yes	2-fold increase at 37°C	Inconsistent	*GAL1* induction impaired in *spt6-1004*
pTET-LYS2	Yes (2/2)	Yes	Decrease	None	*TET* induction impaired in *spt6-1004*

appears that many of these mutagenesis assays do not function properly in the presence of *spt6-1004*, as *GAL1* and *TET* induction are impaired. Overall, the effect of *spt6-1004* on TAM appears to be dependent on the assay used. A contributing factor to this variability between assays may be the effect of *spt6* mutants on the inducible promoters. Alternatively, there may be additional factors that control the degree of TAM that are currently unknown. Therefore, the current standard TAM assays are not sufficient to address the role of Spt6 in TAM. We await newer innovative TAM assays to further pursue this important question in Spt6 biology.

Discussion

In this chapter, I explored a possible role for Spt6 in transcription-associated mutagenesis. To address this question as comprehensively as possible, I employed two published mutagenesis assays as well as one assay which I constructed myself based on data from previous Spt6 experiments performed in our lab. After integrating a well-characterized *spt6* mutation into all of these systems, I optimized a mutagenesis protocol and repeated each assay several times to check whether results were reproducible. My results were highly reproducible and replicated the literature. However, no definitive conclusion could be made regarding the effect of Spt6 on TAM. This was partly due to conflicting results depending on the assay used. It was also due to the confounding effect of an *spt6* mutant on the inducible promoters required for these assays.

As mentioned previously, some of the assay variation in the magnitude of the effect of *spt6* mutants on TAM may be explained by the effect of *spt6* mutations on inducible promoters. It is already known that *spt6-1004* compromises the speed and level of induction of galactose-inducible promoters (Kaplan et al., 2005). Preliminary experiments suggest that *spt6-1004* also inhibits tetracycline-inducible promoter induction (data not shown). This is an important insight that should be taken into account in future Spt6 experiments. Because Spt6 has so many diverse roles in the cell, it is common to see experiments confounded by other unrelated effects of Spt6. Given this and my results, better TAM assays will be required to address the role of Spt6 in this process.

With regards to the *URA3* mutagenesis assay, it is intriguing that *spt6-1004* has different effects at two highly transcribed loci, *CHA1::URA3* and *GLK1::URA3*. As noted earlier, a preliminary result suggests that this may be due to higher transcription at *CHA1::URA3* compared to *GLK1::URA3*. This difference in mRNA levels is consistent with previous analysis of *CHA1* and *GLK1* mRNA levels (Ivanovska et al., 2011). Another possible explanation for the variable results of the *URA3* assay has to do with one of the initial assumptions of the assay; that the degree of nucleosome loss over the *URA3* ORF will be similar to the amount of nucleosome loss over the endogenous gene. In an *spt6* mutant, *CHA1* and *GLK1* have been shown to have very similar levels of Spt6 and RNAPII recruitment and nucleosome loss (indicated by similar ChIP profiles for H2A, H3, and H4) (Ivanovska et al., 2011). Overall, my results highlight the importance of performing these experiments at multiple loci. In the future, it would be useful to develop a method for exploring TAM effects genome-wide.

The experiments presented in this chapter attempted to address an important question regarding the possible role of Spt6 in transcription-associated mutagenesis. Unfortunately the current techniques of the field are not sufficient to provide an answer to this question at this time. Our results indicate that Spt6 can affect the promoter systems being used in these assays, independent of any effect on TAM. However, this work has hopefully laid the groundwork for future TAM experiments. With newer better techniques, we should someday be able to elicit the role of Spt6 in the intersection between transcription, chromatin structure, and DNA damage repair.

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Chapter 4

Summary and Perspectives

Spt6 is a crucial histone chaperone and transcription regulator. As discussed in Chapter 1, the phenotypes of yeast *spt6* mutants suggest that Spt6 has numerous roles *in vivo* and directly interacts with a number of other proteins, including histones and RNA polymerase II (RNAPII) (Adkins and Tyler, 2006; Ardehali et al., 2009; Bucheli and Buratowski, 2005; Carrozza et al., 2005; Chen et al., 2012; Cheung et al., 2008; Chu et al., 2006; Close et al., 2011; Degennaro et al., 2013; Diebold et al., 2010a; Diebold et al., 2010b; Endoh et al., 2004; Formosa et al., 2002; Hartzog et al., 1998; Ivanovska et al., 2011; Jensen et al., 2008; Kaplan et al., 2005; Kaplan et al., 2003; Kiely et al., 2011; Liu et al., 2011; McDonald et al., 2010; Sun et al., 2010; Wang et al., 2013; Winkler et al., 2000; Yoh et al., 2007; Yoh et al., 2008; Youdell et al., 2008). However, the mechanisms of action of Spt6 and its cellular roles are still unclear. In this dissertation, I have described several approaches that I took to investigate the function of Spt6.

In Chapter 2, I described three separate approaches that I used to perform structure-function analysis of Spt6. The first involved random mutagenesis of *SPT6* in an effort to identify novel mutant phenotypes and to correlate phenotypes with the physical location of the mutations. In contrast, the latter two approaches were site-specific mutations, designed to test the roles of the C-terminal SH2 domains and the Spt6 N-terminal domain (NTD) interaction with the transcription factor Spn1/Iws1. Below, I briefly review my results, their implications, and possible future experiments.

For all the *spt6* mutants examined in my dissertation, it would be revealing to perform further functional analysis to understand the effect of mutations in *SPT6* more comprehensively. Functional analysis could include chromatin immunoprecipitation (ChIP) to determine changes in Spt6 recruitment either at highly transcribed genes (for

example, ChIP at *PMA1, CHA1,* and *GLK1*) or genome-wide (ChIP-seq) (Ivanovska et al., 2011). RNA-seq analysis would also be useful to ascertain overall patterns in gene expression changes in *spt6* mutants (M. Murawska, unpublished data). Nucleosome position mapping in *spt6* mutants could be performed as previously described (Degennaro et al., 2013; Ivanovska et al., 2011). Also, Western blot analysis could be conducted to analyze possible histone modification changes in *spt6* mutants (for example, changes in H3K36, H3K4, H3K9, and H3K27 methylation, and H3K14 acetylation) (Begum et al., 2012; Carrozza et al., 2005; Chen et al., 2012; Cheung et al., 2008; Chu et al., 2006; Degennaro et al., 2013; Joshi and Struhl, 2005; Kato et al., 2013; Kiely et al., 2011; Wang et al., 2013; Yoh et al., 2008; Youdell et al., 2008). These proposed experiments would more comprehensively characterize the effect of a given *spt6* mutation on transcription regulation and chromatin structure.

In regards to any possible role in DNA damage repair (DDR), it would be helpful to determine if Spt6 is recruited to double-strand breaks (DSBs). This could be done using Spt6 ChIP performed in currently available systems that generate a single DSB at a known location (see Appendix 1) (Kim and Haber, 2009; Tsukuda et al., 2009). In addition, any newer TAM assays developed in the future (discussed below) may be used to determine if these *spt6* mutations affect DDR and transcription-associated mutagenesis (TAM). Although my work did not confirm a role for Spt6 in DDR at this time, more advanced future techniques and experiments may lead to new insights in this area.

In addition, further analysis of the NTD of Spt6 would be particularly interesting and useful. As discussed in Chapters 1 and 2, the acidic NTD binds Spn1 and nucleosomes exclusively to one another and also diminishes double-stranded DNA (dsDNA) binding

(Close et al., 2011; Diebold et al., 2010a; McDonald et al., 2010). How all of these functions coordinate and relate is still unclear. It may be helpful to further analyze the *spt6-IF, GG, YW, Y* and *W* mutants described in Chapter 2 (as well as *spt6* mutants with alterations in the NTD created by other researchers (McDonald et al., 2010)) and determine their effects on nucleosome positioning (nucleosome position and occupancy assays), Spt6 and Spn1 recruitment (ChIP-seq), histone modifications (Western blot analysis) and dsDNA binding (gel shift assays). Also, the NTD is the one main region of Spt6 for which there is no structural data; future work to solve this structure should shed light on the complex function of this Spt6 domain.

It would also be intriguing to follow up on the DNA-binding properties of Spt6. It has been shown that the core of Spt6 (containing the HtH, YqgF, HhH and DLD domains) is capable of binding dsDNA (Close et al., 2011). It would be useful to further narrow down which domain is necessary and sufficient for this interaction. Also, a key unanswered question is what is the function of the Spt6-dsDNA binding interaction. It will be interesting to investigate if dsDNA binding is required for any or all of the many roles of Spt6. For example, it is unknown whether the DNA binding activity of Spt6 has any effect on Spt6 recruitment to chromatin. Although I suspect that dsDNA binding would be important for the function of a transcription elongation factor and histone chaperone such as Spt6, this has not yet been studied.

In addition, it is also unclear if Spt6 interacts directly with either single-stranded or double-stranded RNA. Gel shift assays could be used to clarify whether Spt6 does in fact bind RNA and, if so, with which domain. If RNA-binding activity is observed, it will be

interesting to explore how this affects the function of Spt6 in transcription, as well as mRNA processing and export.

Regarding the collaborative SH2 domain project described in Chapter 2, a logical next step for this project would be to construct the one additional SH2 truncation mutant which was not studied here: deletion of only the $SH2_1$, the more N-terminal and more standard SH2 domain. Analyzing this mutant would clarify the isolated effect of $SH2_1$ and indicate whether $SH2_2$ retains any function without its $SH2_1$ counterpart. Future work on a deletion of just $SH2_1$ may yield further helpful insights into tandem SH2 domain biology.

As discussed in Chapter 1, the C-terminal $SH2_2$ domain is distinct from other known SH2 domains. Although its overall secondary structure is that of a standard SH2 domain, the $SH2_2$ domain lacks sequence conservation with other SH2 domains and has an unusually shallow binding pocket with the critical binding residues missing (Close et al., 2011; Diebold et al., 2010b; Liu et al., 2011; Sun et al., 2010). Because of this, the Spt6 $SH2_2$ domain was not detected previously based on sequence homology to standard SH2 domains and it is believed to be a member of a novel subfamily of SH2 domains (Close et al., 2011; Liu et al., 2011). This raises the possibility that other non-canonical SH2 domains, similar to the Spt6 $SH2_2$ domain, may exist in yeast or other organisms that have not yet been identified. It would be interesting to examine metazoan genomes for sequences homologous to that of the non-standard Spt6 $SH2_2$ domain to determine if other similar SH2 domains exist and have previously escaped detection. Discovery of other unique SH2 domains would not only confirm the existence of a new subfamily of SH2 domains but would also help further our understanding of SH2 domain biology.

Lastly, given that the SH2 truncation mutants I constructed had such dramatic phenotypes, it may be useful to test them for other phenotypes or defects. For example, do these truncations have any effect on binding Spn1 or histones? Do they affect dsDNA binding or Spt6 recruitment to chromatin? One study has shown that deletion of both tandem SH2 domains decreases Spt6 chromatin recruitment by two-fold (Mayer et al., 2010). However, what is the effect of deleting just one SH2 domain or the other? Answering these questions would provide valuable insights into Spt6 and SH2 domain function.

For the final project of Chapter 2 regarding Spn1, there are a number of structural experiments that would be very helpful in the future. A key experiment is solving the structure of Spt6 bound to histones. This structure would begin to answer many important questions of Spt6 biology such as how many and which histones Spt6 binds, as well as what residues of Spt6 and histones are likely required for their binding interaction. Also, now that a nearly-complete structure of Spt6 is solved using combined data from three independently determined partial structures, it may be possible to determine the structure of Spn1 bound to the entire Spt6 protein, instead of only a small portion of Spt6 (Close et al., 2011; Diebold et al., 2010a; Diebold et al., 2010b; Liu et al., 2011; McDonald et al., 2010; Sun et al., 2010). This would indicate whether Spn1 has any additional interactions with other regions of Spt6. Comparison of an Spt6-histone structure with an Spt6-Spn1 structure would be particularly intriguing and would reveal whether Spt6 undergoes any conformational changes when switching between binding partners. Overall, future structural studies of Spt6, Spn1 and histones are an important next step for determining the details of Spt6 interactions with its binding partners.

In the project described in Chapter 3, I explored a possible role for Spt6 in transcription-associated mutagenesis. Spt6 has previously been shown to have a number of DDR-related mutant phenotypes indicating a role in DDR, and its histone chaperone activity and recruitment to transcribing RNAPII suggests that Spt6 might assist in preventing transcription-associated mutagenesis or TAM. To address this question as comprehensively as possible, I employed all currently available published standard TAM assays as well as one new assay that I constructed myself based on data from previous Spt6 experiments performed in our lab. However, no definitive conclusion could be made regarding the effect of *spt6-1004* on TAM because it caused different effects in different assays.

There is an urgent need in this field is for new and more reliable TAM assays, especially in the case of studying Spt6. One of the main drawbacks of the current TAM assays used is that Spt6 interferes with the required inducible promoters. One approach to avoid this is to use a promoter system that is unaffected by Spt6. Alternatives include steroid-regulated or metal ion-regulated promoters (Gao and Pinkham, 2000; Leskinen et al., 2003; Macreadie et al., 1989). However, to my knowledge, there is no data regarding the effect of Spt6 on these promoters so that would need to be tested beforehand by Northern blot and quantitative real time PCR (qPCR) analysis.

Another approach for improving the current TAM assays would be to construct TAM detection systems at other loci in the genome (for example, insert *URA3* at other highly or lowly transcribed genes or use genes other than *CAN1* and *LYS2* as reporters). In an ideal world, TAM levels would be assayed genome-wide to get a complete picture of the effects of *spt6* mutants on mutagenesis. Recent advances in single-cell sequencing technology could

theoretically be used to examine TAM genome-wide in a population of cells in the future (Hou et al., 2012; Navin et al., 2011; Voet et al., 2013; Wang et al., 2012; Xu et al., 2012; Zong et al., 2012). Whole genome sequencing would also enable researchers to determine the types of mutations generated and whether there is an increase in 2-3 bp deletions which are characteristic of TAM (Lippert et al., 2011; Takahashi et al., 2011). On a broad scale, genome-wide TAM datasets could be compared to histone turnover, nucleosome occupancy, and gene expression datasets genome-wide, perhaps uncovering correlations indicating some of the physiological variables that influence TAM *in vivo* that we are currently unaware of.

Although Spt6 has been the topic of many studies, much of its mechanism and function is still uncertain. The greatest challenge to investigating a role for Spt6 in DDR and TAM is determining whether Spt6 is directly affecting these processes or affecting them indirectly through Spt6-mediated changes in transcription or chromatin. The high level of DNA recombination and the diversity of DDR-related phenotypes of *spt6* mutants strongly suggest that there is some direct or indirect connection between Spt6 and DDR waiting to be discovered (Malagon and Aguilera, 1996, 2001). Overall, despite the experimental challenges, understanding the role of fundamental transcription and chromatin regulators such as Spt6 is crucial to advancing our knowledge of central cellular processes as well as human disease (Bamford et al., 2004; Barnett, 2007; Cygnar et al., 2012; Gallastegui et al., 2011; Griffiths, 2008; Hahn and Young, 2011; Nakamura et al., 2012; Rando and Winston, 2012; Vanti et al., 2009; Winkler et al., 2000; Yoh et al., 2007).

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Appendix 1

Exploring a Role for Spt6 in Homologous Recombination

Attribution of Experiments in Appendix 1

All yeast strain construction, assays, and figures were completed by Erin Loeliger. The *GAL1-HO* strain was kindly provided by the lab of James Haber.

Overview

This appendix summarizes experiments to test for a possible role of Spt6 in DNA damage repair. It focuses on double-strand break (DSB) repair by homologous recombination (HR), the dominant repair mechanism in *S. cerevisiae* (Lisby and Rothstein, 2009). Overall, the *spt6* mutant, *spt6-1004* was found to have no apparent effect on HR by the assay used.

Background

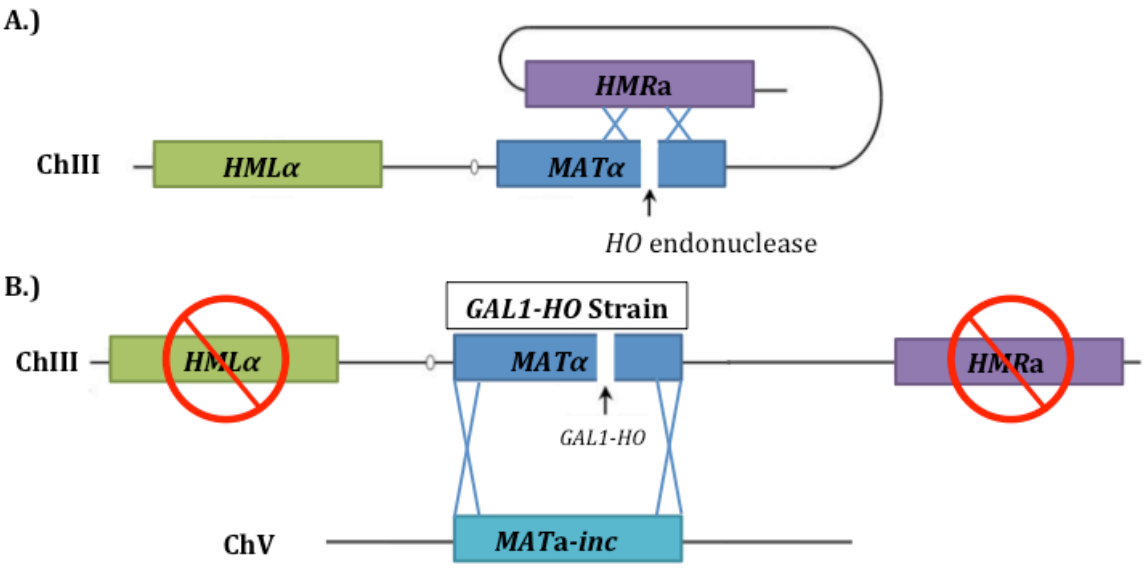
Many chromatin remodelers and histone chaperones have been implicated in DNA damage repair (De Koning et al., 2007; Gospodinov and Herceg, 2013; Groth et al., 2007; Kim and Haber, 2009; Seeber et al., 2013; Tsabar and Haber, 2013). Preliminary data shows that *spt6* mutants are sensitive to the DNA-damaging agent phleomycin (I. Ivanovska, unpublished data; E. Loeliger, unpublished data). Also, the *spt6-1004* mutant interacts genetically with deletion of the *DUN1* gene (I. Ivanovska, unpublished data); Dun1 is a kinase involved in HR, the main DSB repair pathway in *S. cerevisiae* (Chen et al., 2007; Zhou and Elledge, 1993). In addition, *spt6* mutants have increased DNA recombination (Malagon and Aguilera, 1996, 2001). These findings prompted us to ask whether Spt6 is involved in DSB repair through HR, perhaps by affecting chromatin structure near DSBs.

To determine whether Spt6 is required for DSB repair, the *spt6-1004* mutant (known to be sensitive to phleomycin) was assayed for DSB repair efficiency using a specialized yeast strain created by the Haber lab (Kim and Haber, 2009; Tsukuda et al.,

2009). In this strain (referred to here as the *GAL1-HO* strain), expression of the *HO* endonuclease regulated by the *GAL1* promoter produces a single DSB within the *MATα* locus on chromosome III when induced with galactose (Figure A1-1) (Kim and Haber, 2009). This *GAL1-HO* strain lacks both the *HMLα* and *HMR*a loci that normally could repair the *MAT* locus by homologous recombination, resulting in mating type switching. However, this *GAL1-HO* strain has been engineered to have an additional copy of the *MAT***a** locus, *MAT***a**-inc, on chromosome V which allows repair through ectopic recombination. *MAT***a**-inc differs from *MAT***a** only in that it contains a mutation rendering it resistant to *HO* digestion. Thus, once the DSB has been repaired by homologous recombination using *MAT***a**-inc, the strain will not be sensitive to the presence of galactose-inducible *HO*. In this system, viability when grown on galactose is a read-out of DSB repair efficiency.

Using standard techniques, the *spt6-1004* mutation was integrated into the *GAL1-HO* strain described above, replacing wild-type *SPT6* (Rose et al., 1990). These strains were grown in YPLactate medium until log phase and then plated on YPGlucose (no DSB control) or YPGalactose (DSB induction). As controls, this protocol was repeated with the original *GAL1-HO* strain and with an *spt6-1004* strain lacking the *GAL1-HO* system. The number of colonies on galactose compared to the number of colonies on glucose was calculated after five days to provide a functional read-out of DSB efficiency in the cell.

Figure A1-1: System for analysis of DSB repair. A. Schematic of normal mating type switching at the *MAT* locus. The *MAT* locus (dark blue) converts between two alleles, *MATα* and *MAT***a**, by homologous recombination using information from one of two silent mating type cassettes (*HMLα* and *HMR***a**, green and purple). This process is initiated by *HO* which generates a site-specific DSB leading to HR using one of the silent mating type cassettes to repair the *MAT* locus. **B.** The DSB system developed by the Haber lab (Kim and Haber, 2009). In this system, *HO* is controlled by a galactose-inducible promoter. In the presence of galactose, a site-specific DSB is generated. In these cells, however, both silent mating type cassettes have been deleted, preventing normal mating type switching. Instead, an additional mating type cassette, *MAT*a-*inc*, has been inserted on chromosome V (light blue). This cassette is identical to *MAT***a** except for a mutation rendering it resistant to *HO* digestion. If HR is functioning normally, *MATα* will be replaced by *MAT***a**-*inc* and the strain will be unaffected by galactose-inducible *HO*. However, if HR is defective, *MATα* will be left unrepaired, resulting in cell death. **C.** Summary of possible outcomes of assay. (This figure is adapted from Kim and Haber, 2009; Tsukuda et al., 2009).



C.)

Strain	Medium condition	DSB?	Viable?
GAL1-HO, otherwise WT	Glucose	No	Yes
GAL1-HO, otherwise WT	Galactose	Yes	Yes
GAL1-HO, DSB repair defect	Glucose	No	Yes
GAL1-HO, DSB repair defect	Galactose	Yes	No

Figure A1-1: System for analysis of DSB repair (Continued).

Results and Discussion

In my hands, this DSB assay was reproducible and consistent with what was previously observed in the literature (Figure A1-2) (Kim and Haber, 2009). However, there was no significant difference in DSB repair between the *GAL1-HO* and *GAL1-HO spt6-1004* strains. This indicates that *spt6-1004* does not affect DSB repair by HR using this assay.

There are both biological and technical possible reasons why *spt6-1004* showed no effect on HR by this assay. Spt6 is known to affect the magnitude of induction at galactose-inducible promoters (Kaplan et al., 2005). As discussed in Chapter 3, assays relying on such promoters can be drastically compromised by *spt6* mutants, leading to ambiguous or negative results. To check for this, *HO* induction could be monitored by Northern blot analysis and sufficient DSB formation could be detected by Southern blot analysis using a probe corresponding to the *HO* digest site (Keogh et al., 2006; Kim and Haber, 2009).

It is also possible that the effects of *spt6* mutants on DSB repair are allele-specific as has been seen with other phenotypes (Chu et al., 2006). Therefore, it may be informative to assay DSB repair in other *spt6* mutants. In addition, it is conceivable that Spt6 has a role in non-homologous end joining (NHEJ), an alternative pathway for DSB repair in *S. cerevisiae*. To explore this possibility, NHEJ efficiency could be determined by a plasmid repair assay where repair of a linearized plasmid which can only be repaired through NHEJ is measured (Boulton and Jackson, 1996).

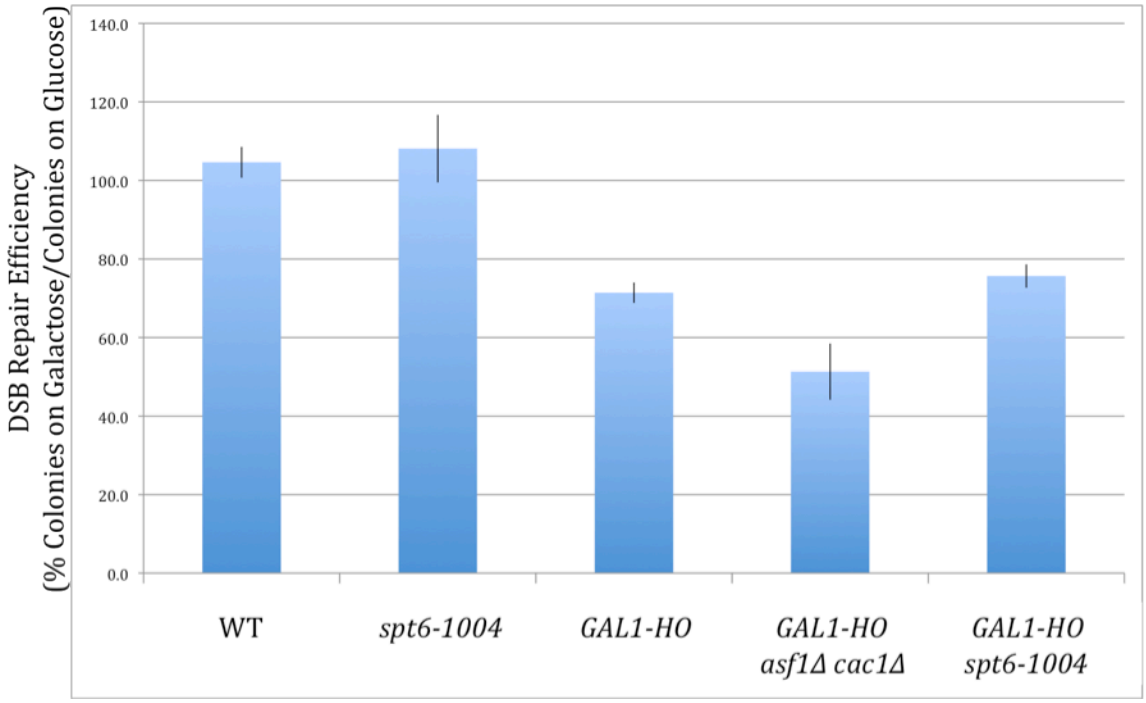


Figure A1-2: DSB assay results for *spt6-1004*. Data shown is the average of three experiments, and the error bars indicate standard error. The positive control (*GAL1-HO asf1Δ cac1Δ*), which contains deletions of two histone chaperones known to affect DSB repair, has an expected decrease in DSB repair compared to the three negative controls (WT, *spt6-1004* (without the *GAL1-HO* system), and *GAL1-HO*). *GAL1-HO spt6-1004* is identical to the *GAL1-HO* negative control.

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Appendix 2

Genetic Interactions Between Dst1 (TFIIS) and Spt6

Attribution of Experiments in Appendix 2

All yeast strain construction and mutant analyses were preformed by Erin Loeliger. The construction of *spt6-14* and *spt6-1004* mutant strains used in Table A2-1 and Figure A2-1 was done by Donald Prather. The information in Figure A2-2A was provided by Marie-Laure Diebold and Christophe Romier. The construction of mutant plasmids used in Figure A2-2B was done by Marie-Laure Diebold and Christophe Romier. All other experiments and figures were completed by Erin Loeliger.

Overview

This appendix summarizes the preliminary evidence for a genetic interaction between Spt6 and Dst1, the *S. cerevisiae* homologue of TFIIS. A deletion of the gene encoding Dst1 (*dst1Δ*) together with *spt6* mutations cause a number of interesting double mutant phenotypes. In fact, *dst1Δ* suppresses the Spt$^-$ phenotype caused by several *spt6* alleles. Our collaborators found evidence for a physical interaction between Dst1 and Spt6, and constructed *spt6* mutations that change amino acids in the suspected Dst1-binding area of Spt6. These mutants have a variety of phenotypes in yeast suggesting that they are in a region important for Spt6 function.

Background

Dst1 is the yeast homolog of the general transcription elongation factor TFIIS (Kipling and Kearsey, 1993; Krogan et al., 2002). Despite the importance of Dst1 in transcription, a *dst1Δ* strain is still viable and causes 6-azauracil sensitivity, consistent with a role in transcription elongation (Kipling and Kearsey, 1993; Krogan et al., 2002; Malagon et al., 2004). The key function of Dst1 is to enable RNAPII to get past elongation blocks by stimulating cleavage of nascent transcripts stalled at transcription arrest sites in order to put the mRNA 3'-hydroxyl back in the RNAPII active site (Krogan et al., 2002; Malagon et al., 2004; Prather et al., 2005b). Dst1 has been shown to bind RNAPII, and *dst1* mutations demonstrate genetic interactions with genes encoding a variety of transcription elongation factors including Spt5, Spt16, Rpb1, Rtf1, and Ctk1 (Archambault et al., 1998; Archambault

et al., 1992; Costa and Arndt, 2000; Davie and Kane, 2000; Hartzog et al., 1998; Jona et al., 2001; Lindstrom and Hartzog, 2001; Malagon et al., 2004; Orphanides et al., 1999; Prather et al., 2005a; Prather et al., 2005b; Wu et al., 1996).

Since both Spt6 and Dst1 are conserved transcription elongation factors, our collaborators were curious what the relationship between them was. Dst1 also has a binding motif very similar to that of Spn1, which is known to bind Spt6, and suggests that Dst1 may have a similar binding affinity for Spt6 (M.L. Diebold and C. Romier, unpublished data). Our collaborators observed that yeast Dst1 was able to bind the Spt6 N-terminal domain, which suggested a functional connection between Spt6 and Dst1 (M.L. Diebold and C. Romier, unpublished data). To follow up on this question, I analyzed *spt6 dst1Δ* double mutants for their phenotypes. In addition, our collaborators identified several conserved regions of the Spt6 N-terminal domain that might be required for Dst1 binding and constructed plasmids containing *dst1* mutants with alterations in these regions. I transformed these mutant plasmids into *S. cerevisiae* to examine their functional consequences *in vivo*.

Results

First, I created a collection of *spt6 dst1Δ* double mutants with different *spt6* mutations, and screened the double mutants for mutant phenotypes. The results are summarized in Table A2-1 and Figure A2-1. Overall, there are a number of genetic interactions between *dst1Δ* and *spt6* mutants. Interestingly, *dst1Δ* suppresses the Spt$^-$ phenotype of suppression of the *lys2-128δ* insertion mutation for some but not all *spt6*

Table A2-1: Genetic interactions between *dst1Δ* and *spt6* mutants. The scoring of the phenotypes for each double mutant and its respective single mutant controls is shown. The yeast strains analyzed are listed in the far left column. The *spt6* mutants used are described in Figure A2-1B. Controls are highlighted in yellow while the double mutants are highlighted in purple. For strains in the upper table, *spt6* mutants were put into *S. cerevisiae* on a *CEN* plasmid. For strains in the lower table, the *spt6* mutants were integrated into the wild-type genomic *SPT6* locus. Each phenotype was given a numerical score of 0 though 5 (0 being no growth and 5 being maximum growth). Genetic interactions are highlighted in red. The "Lys" column indicates the Spt$^-$ phenotype (able to grow on medium lacking lysine (SC-Lys) despite the presence of the *lys2-128δ* Ty LTR insertion allele). The media conditions tested are listed at the top of the table. Phenotype scores are listed for day 3 for all phenotypes except those marked at the bottom by an asterisk (*), which were scored on day 2. Details regarding media conditions used can be found in Hampsey et al., 1997 and in Table 2-4 in Chapter 2. (Abbreviations: WT, wild-type; Lys, SC-Lys; MPA, Mycophenolic acid; HU, Hydroxyurea; Phleo, Phleomycin; MMS, Methyl methanesulfonate; Caf, Caffeine; Can, Canavinine; Dia, Diamide)

Table A2-1: Genetic interactions between *dst1Δ* and *spt6* mutants (Continued).

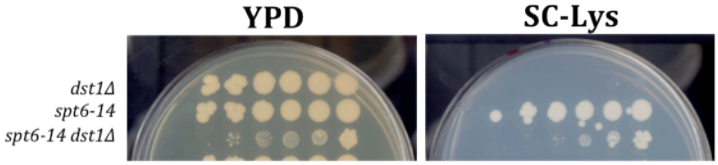
Plasmid-borne *spt6* mutants

	YPD	SC	Lys	16°C	37°C	MPA	HU	Phleo	MMS	UV	Caf	Can	Dia
WT strain	5	5	3	3	5	5	5	3	5	5	3	5	1
dst1Δ	5	5	5	2	5	2	5	3	5	5	2	4.5	1.5
WT SPT6	5	5	4	3	5	5	5	3	5	5	4	5	2
WT SPT6/dst1Δ	5	5	2	3	5	2	5	2	5	5	2	4	2
WT SPT6/dst1Δ	5	5	2	3	5	2	5	2	5	5	2	4	1
spt6-50	5	5	5	2	5	5	2	1	5	5	4	4	1
spt6-50/dst1Δ	4	4	4	2	4	2	2	1	4	4	2	2	0
spt6-YW	5	5	5	3	1	5	2	1.5	5	5	4	3	2
spt6-YW/dst1Δ	4.5	4.5	2	2	1	0	2	1	4.5	4.5	2	1	0
spt6-YW/dst1Δ	4.5	4.5	2	2	1	0	2	1	4	4.5	2	1	0
spt6-W	5	5	1	3	5	5	5	3	5	5	4	4	1
spt6-W/dst1Δ	5	5	1	3	5	2	5	2	5	5	2	4	1
spt6-W/dst1Δ	5	5	1	3	5	3	5	2	5	5	2	4	2
spt6-IF	5	5	5	3	3	5	5	3	5	5	3	4.5	2
spt6-IF/dst1Δ	5	5	4	3	2	2	4	2	5	5	2	3	2
spt6-IF/dst1Δ	5	5	5	3	2	2	4	2	5	5	2	3	2
spt6-GG	5	5	1	3	5	5	5	3	5	5	3	4.5	2
spt6-GG/dst1Δ	5	5	1	3	5	4	4.5	2	5	5	2	4	1
spt6-GG/dst1Δ	5	5	1	3	5	4	4.5	2	5	5	2	4	1

Genomic *spt6* mutants

	YPD	SC	Lys	16°C	37°C	MPA	HU	Phleo	MMS	UV	Caf
WT strain	5	5	0	2	5	4	3	2	5	4	4
dst1Δ	5	5	0	2	5	0	4	2	5	4	2
spt6-14	5	5	5	2	0	4	4	1	5	4	3
spt6-14/dst1Δ	4	4	2	1	1	1	2	1	3	2	2
spt6-1004	5	5	5	2	0	3	4	1	5	4	2
spt6-1004/dst1Δ	5	5	4	2	1.5	0	3	1	4	4	2
			*				*		*	*	

A.)



B.)

spt6 mutant used	Description of *spt6* mutation	Synthetic sick interactions of the double mutant *spt6 dst1Δ*
spt6-50	C-terminal tSH2 truncation	No relevant phenotypes
spt6-YW	Y255A, W257A mutations in N-terminal domain	*dst1Δ* suppresses Spt⁻ phenotype, sensitive to MPA, Canavanine, Diamide
spt6-W	W257A mutation in N-terminal domain	No relevant phenotypes
spt6-IF	I248A, F249A mutations in N-terminal domain	No relevant phenotypes
spt6-GG	G250A, G252A mutations in N-terminal domain	No relevant phenotypes
spt6-14	S952F mutation in the HhH domain	*dst1Δ* suppresses Spt⁻ phenotype, sensitive to HU, MMS, UV
spt6-1004	HhH domain deletion	No relevant phenotypes

Figure A2-1: Genetic interactions between *dst1Δ* and *spt6* mutants. A. Example of *spt6 dst1Δ* spot tests. **B.** Summary of all conditions under which a genetic interaction was observed between *dst1Δ* and an *spt6* mutant. For more details, see Table A2-1. The *spt6-Y dst1Δ* mutant could not be generated for unknown technical reasons and was not tested. (Abbreviations: MPA, Mycophenolic acid; HU, Hydroxyurea; MMS, Methyl methanesulfonate)

mutants. This confirmed our suspicions of a significant genetic interaction between Spt6 and Dst1.

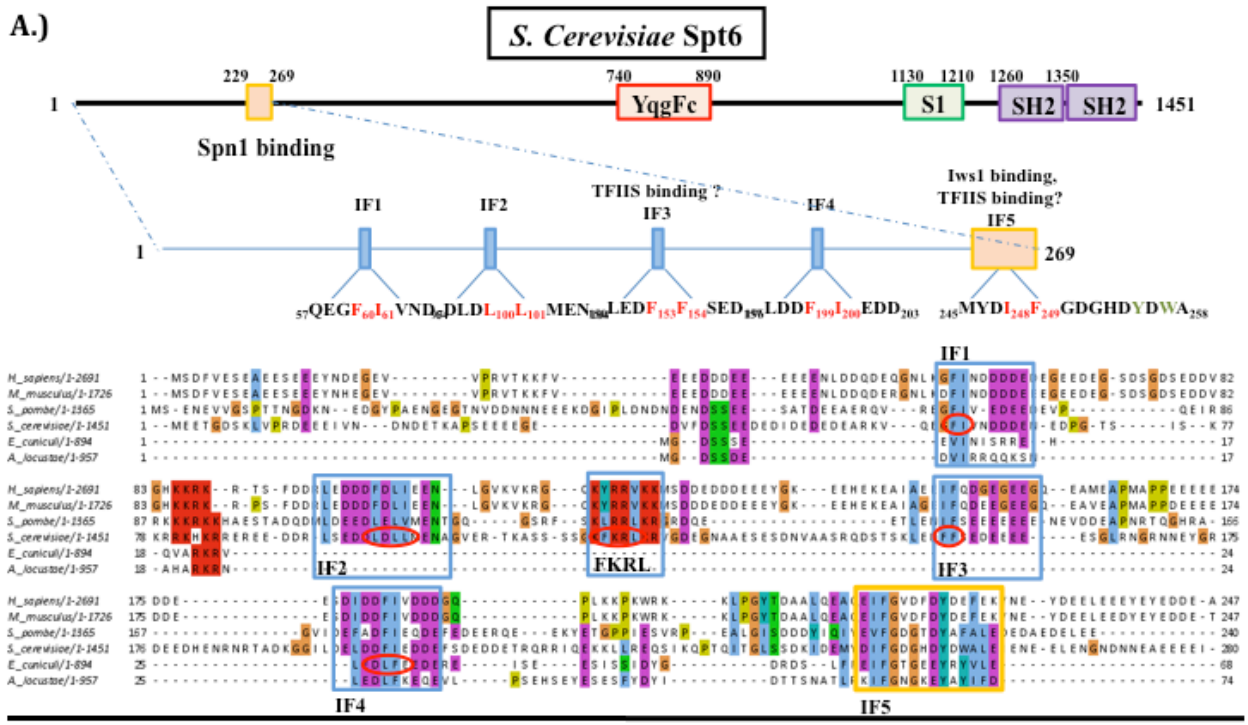
Lastly, I analyzed mutations in the putative Dst1-binding site of Spt6 (Figure A2-2A). These mutations affect some but not all of Spt6 functions (Figure A2-2B). The *spt6-IF1* and *spt6-IF2* mutants, as well as the *spt6-IF* mutant from Chapter 2 *(a.k.a. spt6-IF5*) appear to have the greatest effect of all of these mutations, causing an Spt$^-$ phenotype and cryptic initiation (in the case of *spt6-IF1* and *spt6-IF5*), and an Spt$^-$ phenotype, cryptic initiation, and hydroxyurea sensitivity (in the case of *spt6-IF2*). Altogether, these phenotypes suggest that the altered regions in these mutants are important for Spt6 function.

Discussion

In conclusion, I observed many genetic interactions between Spt6 and Dst1, including the ability of *dst1Δ* to suppress the Spt$^-$ phenotype of some but not all *spt6* mutants. This is the first suppressor of the Spt6 Spt$^-$ phenotype identified to my knowledge, despite numerous mutant hunt attempts to find one.

Interestingly, one of the Dst1 potential binding sites (the region mutated in *spt6-IF* (a.k.a. *spt6-IF5*)) overlaps with the binding site of Spn1 and nucleosomes in the N-terminus of Spt6 (Diebold et al., 2010; McDonald et al., 2010). This suggests that Dst1 binding to Spt6 could potentially interfere with Spt6 binding to Spn1 and nucleosomes. However the implications of this are still unclear and await further study.

A.)



B.)

Mutant	Spt⁻ phenotype	Cryptic initiation	HU sensitive
spt6-IF1	Spt⁻	Cryptic initiation	
spt6-IF2	Spt⁻	Cryptic initiation	Weakly HU^S
spt6-IF3	Weakly Spt⁻		Weakly HU^S
spt6-IF4	Weakly Spt⁻		Weakly HU^S
spt6-IF (a.k.a. spt6-IF5)	Spt⁻	Cryptic initiation	
spt6-FKRL	Weakly Spt⁻		

Figure A2-2: Summary of phenotypes of *spt6* mutants with mutations in the putative Dst1-binding site. A. Diagram of putative Dst1 binding sites on a sequence alignment of the N-terminal domain of Spt6. The red circles indicate residues that have been changed to alanines in each mutant. **B.** Phenotypes of the indicated mutants when put into *S. cerevisiae* on a *CEN* plasmid. (M.L. Diebold and C. Romier, unpublished data)

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